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The Detection of Ciguatera Toxins in NSW Spanish Mackerel

Scomberomorus commerson

Spanish Mackerel

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2024

FRDC Project No **2019-060**

ISBN 978-0-646-89442-3

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In submitting this report, the researcher has agreed to FRDC publishing this material in its edited form.

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2 Acknowledgments

This research would not have been possible without the assistance of numerous individuals, commercial fishing cooperatives and recreational fishing clubs who collected and provided us with samples of Spanish Mackerel caught from the Queensland and New South Wales coastal regions. We thank the following commercial and recreational fishing clubs for their support and active participation in the project: Coffs Harbour Fishing Cooperative, Ballina Fishing Cooperative, Brunswick Heads Cooperative, Clarence River Cooperative, Byron Bay Deep Sea Fishing Club, Mackay Game Fishing Club, Newcastle Neptune's Spearfishing Club, Tweed-Gold Coast Freedivers Club and the Sydney Fish Market.

We thank Trent Alexander, Kyle Johnston and Glen Cuthbert of the NSW Department of Primary Industries Research Angler Program and Shannon White and Shelby Thomas from the Queensland Department of Agriculture and Fisheries monitoring team who provided Spanish Mackerel frames collected from their research.

Samples were collected by the following individuals: Mackay Game Fishing Club: Michael White, Ryan Meads, Ian Meads, Mark Wilcox, Sean Gscheidle, Michael White, Sam Barlow, Noel Lang, Theo Gorgorinis, Noel Lang, Charlie Hayward, Warwick Dunlop, Carl Sheppard, Nathan Ball, Andrew Bond, Grant Pitcher, Joe Collins, Jade Mau, Kody Haynes, Ben Nicholas, Audrina White, Craig Burnett; Ballina Fishing Cooperative: Alister Robertson, Coffs Harbour Fishing Cooperative: Greg Eden, as well as Allen Lo, Kyle Vandermeulen, Philip Sheppard, Jennifer Budworth, Conrad Blignaut, Phillip Hilliard, Jordan Black and Erik Poole.

We would like to thank staff and students from the University of Technology Sydney and the Sydney Institute of Marine Science involved in the analysis of samples, especially Jake Violi, Matt Tesoreiro and David Gertner. We thank staff from the Cawthron Institute who helped analyse the samples using LC-MS. We thank Russell Stafford (QLD Health) for useful discussions and the provision of information on Ciguatera Poisoning (CP) cases in Queensland. We thank Drs Mireille Chinain and Taiana Darius of the Institut Louis Malardé in French Polynesia for their analysis of our samples via the Neuroblastoma (N2a) assay.

3 Abbreviations

CP	Ciguatera Poisoning
CTX	Ciguatoxin
NSW	New South Wales
QLD	Queensland
P-CTX-1B	Pacific Ciguatoxin 1B
RLB	Radio ligand binding
NMR	Nuclear magnetic resonance
MTX	Maitotoxin
MBA	Mouse bioassay
RBA	Receptor binding assay
SFM	Sydney Fish Market
US-FDA	United States Food and Drug Administration
NT	Northern Territory
N2a	Mouse neuroblastoma cells assay
ELISA	Enzyme-linked immunosorbent assay
SPIA	Solid phase immunoassay
QHFSS	Queensland Health Forensic and Scientific Services
LC-MS	Liquid chromatography mass spectrometry
WA	Western Australia

4 Executive Summary

Ciguatera Poisoning (CP) is an illness through the consumption of fish containing naturally occurring toxins, and is considered a high risk for Australian seafood safety. Ciguatoxins (CTXs) are produced by benthic microalgae (*Gambierdiscus spp*). In Australia, CP cases are related to fish caught in Queensland (QLD), Northern Territory (NT) and New South Wales (NSW) waters. Spanish Mackerel (*Scomberomorus commerson*) is the main species that has resulted in CP cases from fish caught in NSW and sub-tropical QLD, and is an important commercial species. An inability to address the risks of CTXs has led to illnesses, with the potential to damage public perceptions of seafood with economic losses to industry. Currently no validated monitoring or measurement methods are available. Prevention methods used internationally are to avoid larger fish of certain species, avoid certain fish species altogether, or avoid fish from certain regions.

A previous project, FRDC 2014-035, set up a facility to determine CTX, and found ~1% of flesh and 7% of liver samples contained detectable CTXs in Spanish Mackerel from NSW waters. A higher proportion of fish from QLD waters contained detectable CTX, with no significant pattern in relation to fish size. As that study tested one analogue of CTXs, Pacific Ciguatoxin 1B (P-CTX1B, or commonly CTX1B), over one year of sampling (n=71 fish), it was recommended that sampling be expanded geographically and temporally to examine biological and environmental variables that could correlate to CP risk. A further recommendation from 2014-035 was that results from liquid chromatography mass spectrometry (LC-MS/MS) be compared against rapid toxicity assays. Discussions with the seafood industry as part of the National Ciguatera Workshop held in 2019 found that rapid, low cost detection of CTXs was a priority. A priority from the NSW RAC in April 2019 for a "A tool to determine the presence of ciguatera in NSW caught Spanish Mackerel".

The present study aimed to: 1) determine industry CTX analysis needs and conduct a viability assessment of CTX measurement tools against these needs; 2) Obtain samples ~300 individual Spanish Mackerel of all sizes caught in industry relevant regions of NSW and QLD waters and measure CTX1B and other available CTXs; and 3) Analyse CTXs in Spanish Mackerel in comparison to biological and environmental variables and 4) Develop recommended options for food safety risk management for Spanish Mackerel to allow for a viable industry while protecting public health.

The analysis of liver and muscle tissue from 249 Spanish Mackerel caught in NSW and QLD waters over 2 fishing seasons (2020/21 and 2021/22) found a lower CTX detection rate using LC-MS in the 2020/21 and 2021/22 seasons in comparison with that of the 2014/15 season. No fish collected during 2020/21 and 2021/22 showed quantifiable levels of CTXs using LC-MS. Using ELISA testing for comparison, from the 2021/22 fishing season, 35 fish of 148 showed a CTX detection but below the level of quantification, and 10 fish had CTX levels that were quantifiable, with the highest at 0.012 µg/kg. Three flesh and liver samples were ≥ 0.01 µg/kg, which is the US FDA CTX guidance level. Fish caught in QLD were considerably more likely to contain CTXs than fish caught in NSW over the 3 fishing seasons. Using the sensitive ELISA method on all fish collected in the 2021/22 fishing season, no fish caught in NSW waters (0 of 32) were found to contain CTXs, whereas 35 of 116 fish (30%) from QLD were found to contain some CTXs, usually below the level of quantification. These CTX+ fish were collected from the vicinity of Fraser Island, Hervey Bay, Rockhampton, Wigton Islands and Coolum.

The lower CTX prevalence in Spanish Mackerel from 2020/21 and 2021/22 fishing seasons in comparison to CTX prevalence in 2014/15, as measured using LC-MS analysis, parallels reports of CP cases, which peaked in 2014-2016 in NSW and QLD, and have been lower in more recent years. Since 2019, 19 CP outbreaks in QLD caused by Spanish Mackerel were reported, and the CTX levels in 10 remaining meal samples of fish were measured using LC-MS by QLD Health. Of fish with CTXs tested, weight and length data were added to our dataset from project 2014-035 to determine the relationship between CTXs and Spanish Mackerel weight or length. With the addition of data, there remains no significant correlation between the prevalence of detectable CTX concentration using LC-MS or ELISA and Spanish Mackerel length or weight.

In an analysis of the environmental, spatial or other correlates of CTXs in Spanish Mackerel and CP cases, an observation was made in relation to annual cyclone frequency and intensity and annual CP cases in QLD and

NSW with a lag time of ~1 year, a relationship that has been previously noted in a study in the Pacific. This requires further analyses to determine its consistency.

From the comparison of CTX detection methods, results from the enzyme-linked immunosorbent assay (ELISA) were well correlated with those from either LC-MS or neuroblastoma cell-based assay (N2a) (r^2 of 0.68 and 0.98, respectively). However, the ELISA and the N2a assay were more sensitive and able to detect the presence of lower level CTXs that were below the level of detection of the LC-MS method. The ELISA test was found to be potentially viable for use at a central site with laboratory facilities. However, CTX extraction from fish tissue currently requires time frames of 6-12 h and complex laboratory equipment to complete. Therefore it is not currently suitable to be performed on site or rapidly. Further development of fish tissue extraction methods would be necessary to determine if ELISA or other more rapid methods are suitable for inclusion in the suite of provisions for CP risk management. Based on our new data, we recommend that current CP regulations remain in place. Recommendations are given for future approaches, including those relevant to public health, analytical advances, and additional environmental and fisheries studies.

Keywords

Spanish Mackerel, *Scomberomorus commerson,* Ciguatera Poisoning, ciguatoxins, toxicology, LC-MS

5 Introduction

5.1 Ciguatera Poisoning

Ciguatera Poisoning (CP) is a significant safety concern in some Australian seafood (Sumner, 2011) and a prevalent global issue associated with fish consumption (Friedman et al., 2008). Globally, it affects 50,000 to 500,000 people annually (Friedman et al., 2017) and is caused by the ingestion of fish containing toxic levels of ciguatoxins (CTXs) (Hamilton et al. 2010).

CTXs are primarily produced by microalgae species of the *Gambierdiscus* genus (Chinain et al., 1997; Holmes, 1998; Chinain et al., 1999; Chinain et al., 2010; Rhodes et al., 2010; Fraga et al., 2011; Holland et al., 2013) and accumulate in the food chain, particularly in carnivorous reef fish (Murata et al., 1990a; Lewis et al., 1991; Lewis & Holmes, 1993; Vernoux & Lewis, 1997; Lewis et al., 1998; Yasumoto et al., 2000; Pottier et al., 2002; Pottier et al., 2003). These toxins activate sodium channels in nerve cells (Lewis et al., 1992; Mattei et al., 1999; Lewis et al., 2000, leading to various gastrointestinal and neurological symptoms in humans with severe cases even affecting the cardiovascular system.) (Figure 1). Diagnosing CP is challenging due to over 175 documented symptoms (Sims, 1987) , which can vary based on portion size (Sims, 1987), individual susceptibility, age (Bagnis et al., 1979; Glaziou & Martin, 1993), geographical region (Lewis et al., 2000; Dickey, 2008). and potential overlap with other illnesses.

CP cases are increasing globally, with a 60% rise in the Pacific region over the past decade (Farrell et al., 2017). Regional differences in CTXs highlight the importance of characterizing toxins from different areas. Understanding CTX accumulation patterns in various fish species can aid in prevention. However, accurate identification of specific CTX congeners is essential to comprehensively assess CP risks locally.

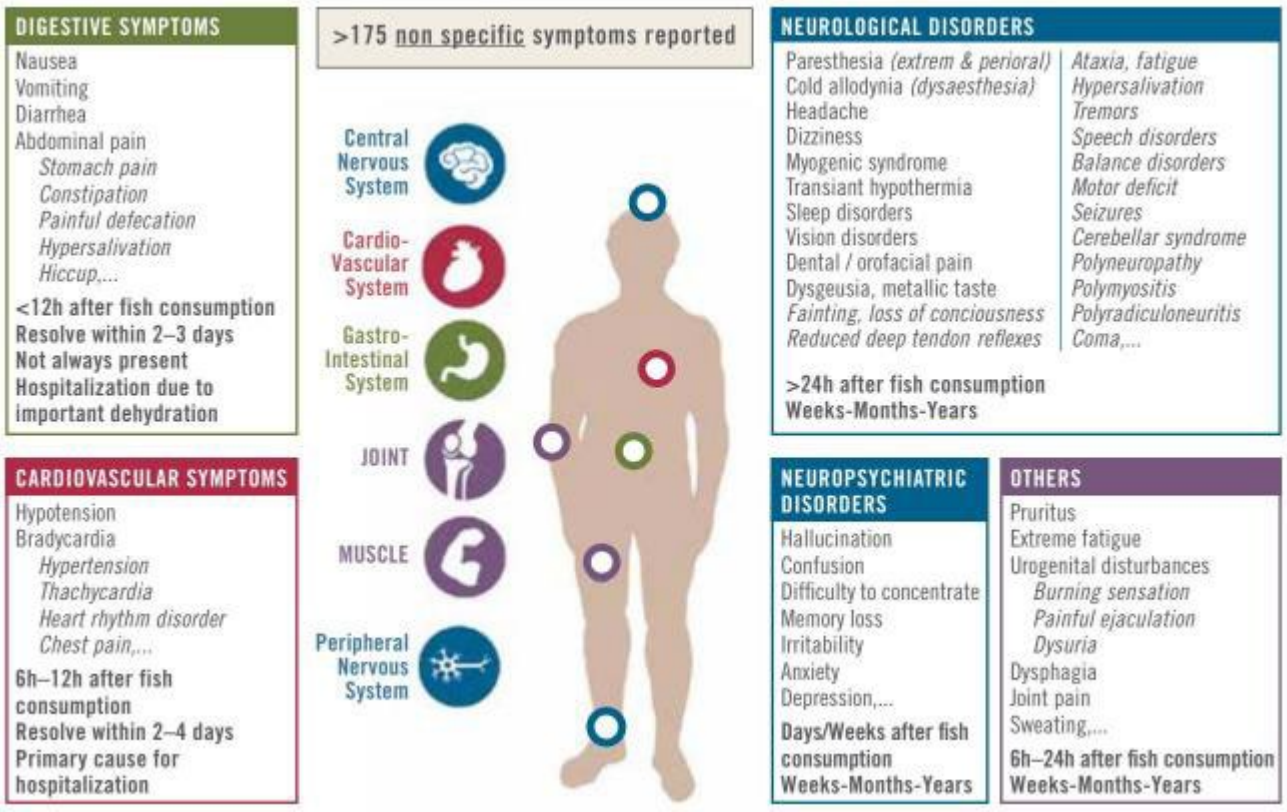


Figure 1. Symptoms connected with ciguatera intoxication. (FAO/WHO 2020).

5.1.1 Chemistry of CTXs

CTXs are cyclic polyether ladders with remarkable thermostability and liposolubility. They have been extracted from various fish species and different *Gambierdiscus* strains (Table A1). These toxins are categorized into P-CTXs (from the Pacific Ocean), C-CTXs (from the Caribbean region), and I-CTXs (from the Indian Ocean) based on their origin and structural distinctions.

Within P-CTXs, there are two main types: type I with 13 rings and 60 carbon atoms (Murata et al., 1990a; Lewis et al., 1991; Lewis & Holmes, 1993; Yasumoto et al., 2000), exemplified by CTX1B (Murata et al., 1990a, Murata et al., 1990b; Lewis et al., 1991) and type II with similar features, represented by CTX3C (Legrand et al., 1998) (Figure 2).

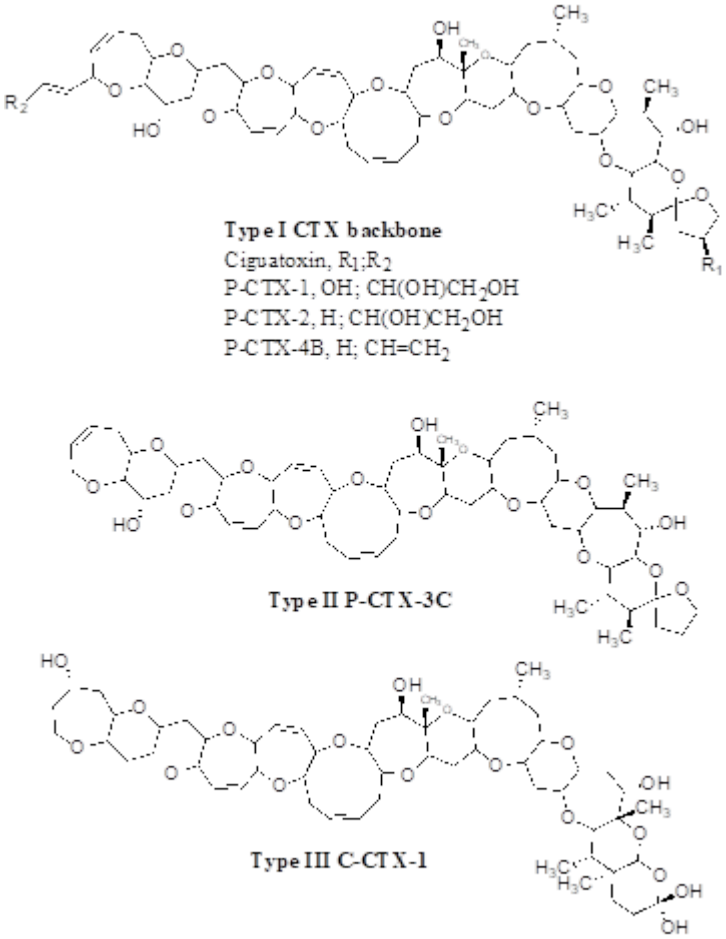


Figure 2. Structure of Ciguatoxins (CTXs). P-CTX-1, P-52-EPI-54-DEOXY-CTX-1B (FORMERLY KNOWN AS CTX-2) and C-CTX-1 were derived from fish and P-CTX-3C and P-CTX-4B were derived from *Gambierdiscuss* spp. (Kohli et al., 2015).

Additionally, 52-epi-54-deoxy-CTX-1 (formerly known as CTX-2) and 54-deoxy-CTX-1B (formerly known as CTX-3), derived from dinoflagellate CTXs, CTX-4A and CTX4B (Lewis & Holmes, 1993; Yasumoto et al., 2000), have variations in their structures, affecting toxicity. Type II P-CTXs include 49-epi-CTX-3C and M-seco-CTX-3C isolated from a *Gambierdiscus* sp. (Satake et al., 1993) and *G. polynesiensis* (Chinain et al., 2010). New variants, such as 2,3 dihydroxyCTX3C and 51-hydroxyCTX3C, have also been identified from Moray eel (Satake et al., 1998).

Caribbean CTXs, larger than P-CTXs, have 14 rings and 62 carbon atoms (Vernoux & Lewis, 1997; Lewis et al., 1998; Pottier et al., 2002; Pottier et al., 2003). Numerous congeners of C-CTXs have been isolated from carnivorous fish (Vernoux & Lewis, 1997; Lewis et al., 1998; Pottier et al., 2002; Pottier et al., 2003). Unlike P-CTXs there have been no reports of C-CTXs being originating from *Gambierdiscus* spp. However, recently *G. excentricus* has been identified as a major CTX producer in the Caribbean (Fraga et al., 2011) and CTXs from this strain are being characterised.

I-CTXs from the Indian Ocean have higher molecular ion masses than P-CTXs and C-CTXs. Four types (I- CTX-1, I- CTX-2, I-CTX-3, I-CTX-4) have been identified but await structural elucidation (Hamilton et al., 2002a; Hamilton et al., 2002b). Toxicity varies among CTX congeners as observed in mouse bioassays (MBA), but further validation is needed (Table A1). Importantly, understanding these structural distinctions is essential for assessing the risks posed by different CTXs.

5.1.2 Detection of CTXs in Seafood

CP cases primarily occur in mid-latitude tropical and sub-tropical zones, reflecting the distribution of *Gambierdiscus* (Kohli et al., 2015). However, CP has been reported in non-endemic areas due to seafood imports of susceptible species (Glaziou & Legrand, 1994; Ting & Brown, 2001). While most studies have focused on reef fish, toxin accumulation has been observed in various species such as eels, sea cucumbers, starfish, seals, and jellyfish (Kohli et al., 2015).

Local knowledge in small island nations often guides safe fish consumption. However, a study in French Polynesia found CTXs in supposedly safe-to-eat fish (Darius et al., 2007). Experimentally, CTX toxin profiles and structures have been determined using chromatographic techniques, nuclear magnetic resonance (NMR), and radio ligand binding (RLB) (Murata et al., 1989; Murata et al., 1990a; Lewis et al., 1991; Satake et al., 1996; Hamilton et al., 2002a; Hamilton et al., 2002b). These methods are costly and not practical for routine testing. Purified and certified CTX standards are limited, hindering accurate quantification.

Various biological assays, such as the MBA and enzyme-linked immunosorbent assay (ELISA), have been developed to detect ciguateric fish. While MBA remains widely used, it has limitations. ELISA offers higher throughput but has produced false results (Hokama, 1990; Campora et al., 2008a; Bienfang et al., 2011). Though, when a different approach to produce antibodies was tried, no cross-reactivity was observed with other marine toxins (Tsumuraya et al., 2018; Tsumuraya & Hirama, 2019). These results led to the development of a new kit named “CTX-ELISA 1B” (Fujifilm Wako Corporation, Osaka, Japan) based on a fluorescent ELISA assay. Since the results obtained with this strategy were promising, the same antibodies were used to develop biosensors which have a limit of detection ten times lower than the United States Food and Drug Administration (US FDA) guidance threshold of 0.01 µg/kg (Leonardo et al., 2020, Campàs et al., 2022, USFDA, 2011). While these tools are portable and user-friendly, the protocol for CTXs detection still necessitates a lengthy extraction process from fish flesh. Other assays like sodium channel binding mouse neuroblastoma cells (N2a) (Manager et al., 1993; Viallon et al., 2020) and receptor binding assay (RBA) (Dìaz-Asencio et al., 2018; Hardison et al., 2016) have shown promise but can't quantify specific CTX congeners. LC-MS analysis is crucial for this purpose, but analytical challenges include the lack of purified standards and the presence of multiple CTX analogues in fish specimens (Endean et al., 1993; Vernoux & Lewis, 1997).

5.2 CP in Australia

CP is a concern in the warmer waters of Australia, primarily along the coastlines of the Northern Territory (NT), Queensland (QLD) and south to Byron Bay in NSW (~28°S). There are no confirmed reports of CP from Western Australia (WA). Most CP outbreaks are linked to fish caught in QLD and the NT, with Spanish Mackerel being the most frequently implicated species (Gillespie et al., 1986; Farrell et al., 2016a). Until 2014, cases of CP in NSW, Victoria, or other southern states were usually traced back to fish from QLD, the NT or imported fish (Farrell et al., 2016a).

Approximately 200 fish and invertebrate species may be involved in CP outbreaks, although precise figures are challenging to determine (FAO, 2020; Kohli et al., 2015). While many implicated species are carnivorous, herbivorous species have also been linked to CP outbreaks. (Friedman et al., 2017) Species like Amberjack (*Seriola* spp.), Wrasse (*Cheilinus* spp.), and Trevally (*Caranx* spp.) are common vectors of CTXs in the Pacific region (Lewis, 2001; Stewart et al., 2010) (Table A5).

In NSW, confirmed CP cases related to Spanish Mackerel consumption from NSW waters have been reported in several locations, including Brunswick Heads in 2002, Evans Head in February 2014 (4 people), Scott's Head in March 2014 (9 people), and South West Rocks in April 2015 (4 people). These cases involved classic CP symptoms, and many required hospitalization, with at least one victim disabled for an extended period (Farrell et al., 2016a). P-CTX-1B was detected via LC-MS/MS in Spanish Mackerel samples during these outbreaks. Additionally, suspected CP outbreaks in 2005 and 2009 in NSW were linked to fish from Fiji and QLD, respectively, but lacked chemical analysis to confirm P-CTX-1B presence. The NSW CP cases in 2014- 2015 mark the southernmost confirmed sources of CP in Australia (Farrell et al., 2016a).

5.3 Management of CP

The US Food and Drug Administration (FDA) has recommended a guidance level for Pacific CTX-1B in fish flesh of less than or equal to 0.01 ppb CTX equivalent (0.01 µg kg^{-1} CTX) (USFDA, 2011). Due to the absence of rapid and cost-effective screening tests for CTXs, health authorities worldwide have typically issued guidelines to prevent high-risk fish from entering the commercial market to reduce the risk of CP (Stewart et al., 2010). It is generally believed that the size or age of certain fish species may be related to the levels of CTXs found, as these toxins can accumulate over time.

Relatively few studies have directly explored the relationship between fish size and CTX presence, with variable results. In a Japanese study, a positive relationship was observed between size and toxicity in several fish species, including *Lutjanus monostigma* (Onespot Snapper, Figure A1), *Epinephelus fuscoguttatus* (Flowery Rockcod, Figure A2), *Lutjanus bohar* (Red Bass, Figure A3), and *Variola louti* (Yellowedge Coronation Trout, Figure A4) (Oshiro et al., 2010). Another study involving Great Barracuda (*Sphyraena barracuda*) found toxic samples, but no clear correlation between fish size/weight and toxicity (Dechraoui et al., 2005). These findings indicate mixed results in the few studies that have directly examined the relationship between fish size and CTX presence (Figure A5).

In Australia, guidelines to prevent high-risk fish from entering the market are provided by the Sydney Fish Markets (SFM) (Table A3 and A4), the country's largest domestic fish distributor (Stewart et al., 2010). Queensland (QLD) and Northern Territory (NT) authorities also follow these guidelines, and CP cases are notifiable conditions in QLD (QLD Health, 2015). The guidelines are based on the observation of outbreaks and illnesses rather than studies relating CTX levels in high risk fishes. In Queensland, QLD Health has established protocols for collecting epidemiological related information (patient symptoms, suspected fish details) and samples for quantification of P-CTX-1, 2, and 3. However, further research is needed to assess and mitigate the risk of CP in Australia.

6 Background to this Project

This project was initiated in response to a request from the NSW Fisheries Research Advisory Committee in April 2019 to develop a tool for detecting ciguatera in Spanish Mackerel caught in New South Wales (NSW). This request followed a National Ciguatera Fish Poisoning Science Workshop held in March 2019 at the University of Queensland, sponsored by SafeFish, and attended by various stakeholders, including the Professional Fisherman's Association (Tricia Beatty) and Sydney Fish Markets (Erik Poole). The workshop led to the development of national ciguatera management and research strategy (SafeFish, 2019), as CTX risks in fish have resulted in illnesses and pose threats to seafood safety and industry.

A prior project, FRDC 2014-035, established the first facility to detect CTX in NSW and found detectable CTXs in approximately 1% of flesh samples and 7% of liver samples from Spanish Mackerel from NSW waters, with no clear pattern related to fish size. This study focused on one CTX analogue, P-CTX1B, and analysed 71 fish samples collected over one year. To address the need for comprehensive data on CP food safety risk, discussions with the seafood industry and outcomes from the National Ciguatera Workshop emphasized the importance of rapid, low-cost CTX detection technologies. The project also recommended comparing results from liquid chromatography mass spectrometry (LC-MS/MS) with rapid toxicity assays, which evaluate toxicity from CTX analogues besides P-CTX1B.

In collaboration with the NSW fishing industry and seafood safety regulators, this project aims to investigate available CTX detection methods and determine the prevalence of CTX in Spanish Mackerel, as well as the factors influencing it. The goal is to enhance CTX management in NSW. Additionally, companion projects are in progress to collect Spanish Mackerel samples from the recreational fishing community in NSW and QLD, funded by the NSW Recreational Fisheries Trust. Furthermore, UTS is conducting an Australian Research Council funded Linkage project on Ciguatera Poisoning, exploring the location of CTX-producing *Gambierdiscus* species in Queensland waters in association with reported cases of CP.

7 Objectives

The present study was formulated in consultation with the Sydney Fish Market, the Professional Fishers' Association, the NSW Food Authority, the FRDC in particular the NSW RAC, and members of the Recreational Fishing community. It was informed by the views of participants in the National Ciguatera Fish Poisoning Science Workshop held in March 2019.

The objectives of the project are:

1) Determine industry CTX needs and conduct a review of available CTX measurement tools (including cell based assays, ELISA kits, and LC-MS) against these needs. Conduct an assessment of the currently available screening tools to determine which, if any, hold promise for industry use. Conduct a viability assessment for how a tool might be used in industry or, if none of the currently available tools are appropriate, make recommendations for future activities to develop a rapid screening tool that meets industry needs.

2) Obtain samples of flesh and liver from ~300 individual Spanish Mackerel of all sizes caught in industry relevant regions of NSW waters over a period of 2 years and collate fish length, weight, sex and site information, with the participation of the Sydney Fish Market and commercial fishing cooperatives. Obtain samples from any individual Spanish Mackerel associated with illnesses in NSW or QLD. Measure CTX1B and other available CTX analogs using best practice methods identified in Objective 1.

3) Conduct statistical data analyses of all available data on CTX concentrations in Spanish Mackerel in comparison to biological and environmental variables. Develop recommended options for food safety risk management for Spanish Mackerel in NSW that will allow for a viable industry while protecting public health.

8 Review of ciguatoxin analytical techniques and industry needs

8.1 Introduction

CP is the most common marine biotoxin food poisoning in the world and accounts for nearly half of all seafood related illness outbreaks in Australia (OzFoodNet data, 2001-2015). While over 180 species of fish are known to be carriers of CTXs, Spanish Mackerel (*Scomberomorus commerson)* is one of the species more frequently implicated in CP in Australia (Farrell et al., 2016a; Gillespie et al., 1986). The vast majority of CP outbreaks involving Spanish Mackerel have historically been associated with fish caught in QLD and the NT (Gillespie et al., 1986), but more recently, 31 cases of CP have been reported in NSW (first reported in 2014, Farrell et al., 2016a), with consequences for the local Spanish Mackerel fishing industry. Current management of CTX in Spanish Mackerel is not based on active testing of CTX, but rather involves the rejection of fish that exceed 10 kg in weight (or 8 kg if headed & gutted, Sydney Fish Market guidelines, 2015). In the most comprehensive screening of NSW caught Spanish Mackerel to date (71 fish), CTX was detected in the flesh of one fish and the liver of 5 fish, providing an estimated prevalence of CTX of 1-4% in flesh and 1-12% in liver tissues (based on 95% confidence interval, Kohli et al., 2016, 2017). Toxin levels found were <0.1 μg kg^{-1} - 0.13 μg kg^{-1} in muscle tissue, and <0.4 μg kg^{-1} - 1.39 μg kg^{-1} in liver tissue, which is up to 139 times higher than the US FDA guidance level. These results indicated that liver tissue had a significantly higher concentration (~5 fold) of P-CTX-1B. Based on these results, there is a strong interest from both industry and regulators in refining the current management of CTX in Spanish Mackerel by collecting additional prevalence data and investigating CTX analytical techniques to detect ciguatoxins in fish before market.

8.2 NSW commercial Spanish Mackerel fishery

The NSW Spanish Mackerel fishery is seasonal, targeting fish on trolled lines during the late summer – autumn months when fish migrate into NSW waters from QLD. The estimated catch in recent years has been ~10 t, down from 27 t in 2015 (pers. comm. Tricia Beatty, NSW PFA). The majority of Spanish Mackerel are caught from Forster (~32° S) and further north to the QLD border (Kohli et al., 2016). Fishers operate in small individual vessels (5-9 m) with limited crew (1-2 people), targeting <10 kg Spanish Mackerel. Larger fish, which are often the majority of the catch, are discarded in compliance with Sydney Fish Market CP guidelines. Fishers operate from the early hours of the morning (4 am) until midday- early afternoon, when fishers race back to shore to weigh their catch and send it to the Sydney Fish Market. A single truck operates along the NSW east coast, collecting the catch from fishing cooperatives and individual fishers en-route to market. The truck arrives at the Sydney Fish Market around 1-2 am, with the auction of fresh fish commencing at 5:30 am. The fish are kept fresh on ice and are sold on the same day of arrival (the day after they are caught), as there is no market demand for frozen Spanish Mackerel (pers. comm. Tricia Beatty, NSW PFA). The combination of a small boat fishery, short supply chain and lack of a centralised collection point prior to market severely limits the options for timely testing and release of product before auction.

8.3 Ciguatoxins

Pacific ciguatoxins (P-CTXs) are the only CTX group reported in Australia to date. The biotransformation of P-CTX-4B (Pacific ciguatoxin type 4B) to P-CTX-1B represents a ten-fold increase in toxicity (Lehane and Lewis, 2000), takes place in the presence of fish enzymes and also human liver enzymes (Ikehara et al., 2017). Indeed, of the 22 different Pacific ciguatoxins with different toxicities identified to date (summarised in EFSA, 2010; Pasinszki et al., 2020), P-CTX-1B is the most potent, causing the onset of CP at as little as 0.1-
1.0 μg kg^{-1} (Hossen et al., 2015; Lehane and Lewis, 2000). It should be noted that this level is not an official lowest adverse effect level and is based on the current scientific consensus of available data. It appears highly

likely that CTXs can bioaccumulate in the human liver if exposed to consecutive subacute doses of CTX (Hamilton et al., 2010; Ikehara et al., 2017), leading to United States Food & Drug Administration (FDA) to recommend (not legally binding) a safety factor of 10, bringing the guidance level to 0.01 µg P-CTX-1B equivalents/kg.

CTXs cannot be removed from fish through cooking or freezing and their presence cannot be detected by smell, taste or odour. A number of basic indigenous tests exist, but their accuracy is doubtful at best (section 5.1.2 and 8.7). Even French Polynesian fishers well experienced with CP, using the stiffness and bleeding characteristics of caught fish as guidance, could only distinguish toxic from non-toxic fish with unsatisfactory reliability (Darius et al., 2013). Instead, the detection of ciguateric fish relies on sophisticated analytical techniques that require the extraction of CTX from the fish tissues prior to analysis.

Quantifying extremely low concentrations of highly potent CTX in fish tissues presents a considerable analytical challenge. Combined with the global shortage of purified and certified CTX standards, progress in the development of rapid analytical techniques has been slow. However, significant advances have been made in the detection of CTX in recent years, including functional biological assays and liquid chromatography techniques paired with mass spectrometry (summarised below).

Implementing these techniques to develop a monitoring framework is difficult. This is partly due to limitations of certain analytical techniques, as well as a lack of knowledge around the prevalence of different CTX analogues in fish of different species, sizes and localities (Yogi et al., 2011). To date, none of the currently available CTX analytical methods have been reported to have undergone single- or multi-laboratory validation (WHO, 2020). We here summarise the advantages and disadvantages of currently available CTX detection and quantification methods to explore their use for monitoring of CTX in NSW Spanish Mackerel.

8.4 Analytical targets & toxin standards

The Australian Ciguatera Fish Poisoning Research Strategy summarises the occurrence of CTX in Australian fish to recommend specific CTXs for analysis (SafeFish, 2019). P-CTX-1B is the prime analytical target in Australia, as it has been reported from almost every confirmed CP case in Australia where meal/fish remnants were available for testing (Farrell et al., 2016; Hamilton et al., 2010, and QLD Forensic & Scientific Services data). Where the analytical technique allows and purified CTX standards are available, quantification of 52-epi-54-deoxy-CTX-1B (formerly known as CTX-2) and -3 is also recommended, as 52-epi-54-deoxy-CTX-1B (formerly known as CTX-2) was the major CTX implicated in a fatal case of ciguatera in Queensland, along with potential involvement of its epimer 54- deoxy-CTX-1B (formerly known as CTX-3) (Hamilton et al., 2010). In the Pacific region, P-CTX-4A and -4B have been also been reported in fish (Yogi et al., 2014), where they are thought to be contributing to total toxicity together with other CTXs. Since no standards are available for these CTX analogues, and their potency is considerably lower than that of the other CTXs mentioned above (EFSA, 2010), these analogues were considered as emerging analytical targets. However, it is noteworthy that Ikehara et al. (2017) recently demonstrated the conversion of P-CTX-4A & -4B to the much more potent P-CTX-1B in the presence of a human liver enzyme. This suggests that P-CTX-4A & -4B may contribute more to total sample toxicity than previously thought.

The lack of available CTX standards has long been a major hurdle to the development and implementation of analytical techniques. Only synthesised P-CTX-3C is currently commercially available (Wako Chemicals, $500 for 100 ng) and purified standards of other P-CTXs are only shared by select research groups in small quantities for collaborative research projects. To give an example of the effort involved in isolating purified CTX materials from fish tissues, processing of 48 kg of moray eel viscera by Richard Lewis at the University of Queensland only yielded 100-490 µg of P-CTX-1, -and analogues ie 52-epi-54-deoxy-CTX-1B (Lewis et al., 1991).

8.5 CTX regulations & guidelines

The European Union Directorate General for Health and Food Safety requires that fishery products containing

CTXs should not be placed on the market. However, no regulatory limits and no analytical requirements have been given. There currently is no CODEX standard for CTXs, with the 2018 combined WHO/FAO expert meeting on CP concluding that currently available data are insufficient to conduct a risk assessment of CTX in food (WHO, 2020). The European Food Safety Authority's panel on Contaminants in the Food Chain (CONTAM) estimated that P-CTX-1 levels below 0.01 µg/kg should not have any negative effects in sensitive individuals (EFSA, 2010). Similarly, the US Food & Drug Administration (FDA) has established the identical level as a guidance level for P-CTX-1. However, these values are proposed as recommendations/guidance and are not legally enforceable.

8.6 Extraction of CTXs from fish flesh

All currently available CTX analytical techniques (described below) require a sample preparation step aimed at separating (extracting) highly fat soluble CTXs from the fish tissue. This critical preparation step is designed to separate the CTX from any compounds in the fish tissue (matrix) that might interfere with analysis, such as fats and proteins. CTXs are strongly bound to the fat molecules in the fish tissue and sample preparation procedures typically require several steps to separate the bound CTX from the fish flesh before they can be analysed. Current extraction protocols are time consuming and take anywhere from 6.5 hours to multiple days per sample to complete, depending on extraction protocols and desired final sample purity. These processes include tissue homogenisation (grinding and mincing), extraction (getting solid bound CTX into a liquid solution), partitioning (removal of solids and undesirable contaminants) and defatting of the sample with various sequentially applied solvents (see flowchart in Figure 3). A subsequent step concentrates the sample by evaporating the solvent to yield a crude extract. Most analytical techniques, including liquid chromatography-mass spectrometry (LC-MS), require subsequent procedures to further clean up the extract through solid phase extraction (SPE) cartridges (summarised in detail in Harwood et al., 2017; Pasinszki et al., 2020). Extraction protocols have been consistently improved (historically extractions took >1 week) and tweaked for tissues of different fish species and various sample types, but remain the major time limiting step in CTX analysis. Depending on the assay type, several different extraction protocols exist, and different variations of these procedures are often employed between laboratories to yield maximum CTX recovery from specific fish tissues.

Lewis et al. (2009) developed a CP extraction method for LC-MS analysis (CREM) that requires about 2 g of fish tissue for successful extraction of P-CTX-1, and analogues ie 52-epi-54-deoxy-CTX-1B. This CREM technique was subsequently improved by Stewart et al. (2010) and Meyer et al. (2015) and takes about 6.5 h per sample. The SPE final clean up steps are time consuming, as the extract has to slowly pass through a series of individual SPE cartridges. Analysing the crude extract without SPE steps would significantly shorten the time required for extraction, but manipulating or omitting specific extraction steps carries the risk of not extracting sufficient CTXs to detect their presence (depending on the limit of detection of the chosen method) or introducing impurities that could interfere with the selected assay system. The CREM and LC-MS protocol is sufficient to allow LC-MS to detect CTX below what is commonly considered the lowest adverse effect level of 0.1 µg P-CTX-1B/kg, with a limit of detection of 0.03 µg P-CTX-1/kg (Stewart et al., 2010).

Compared to fish flesh, blood presents a much simpler matrix that does not require as many extraction steps and generally yields higher toxin recoveries (Dechraoui et al., 2007; Mak et al., 2013; O'Toole et al., 2012). For cell line-based assays, extraction of CTX from the blood only requires a single solvent extraction step to remove proteins, followed by centrifugation and solvent evaporation to concentrate the CTXs (Dechraoui et al., 2007; O'Toole et al., 2012). However, LC-MS techniques to quantify ciguatoxins in blood still require a more purified extract and follow the lengthy extraction procedures like the one outlined in Figure 3 below (Mak et al., 2013).

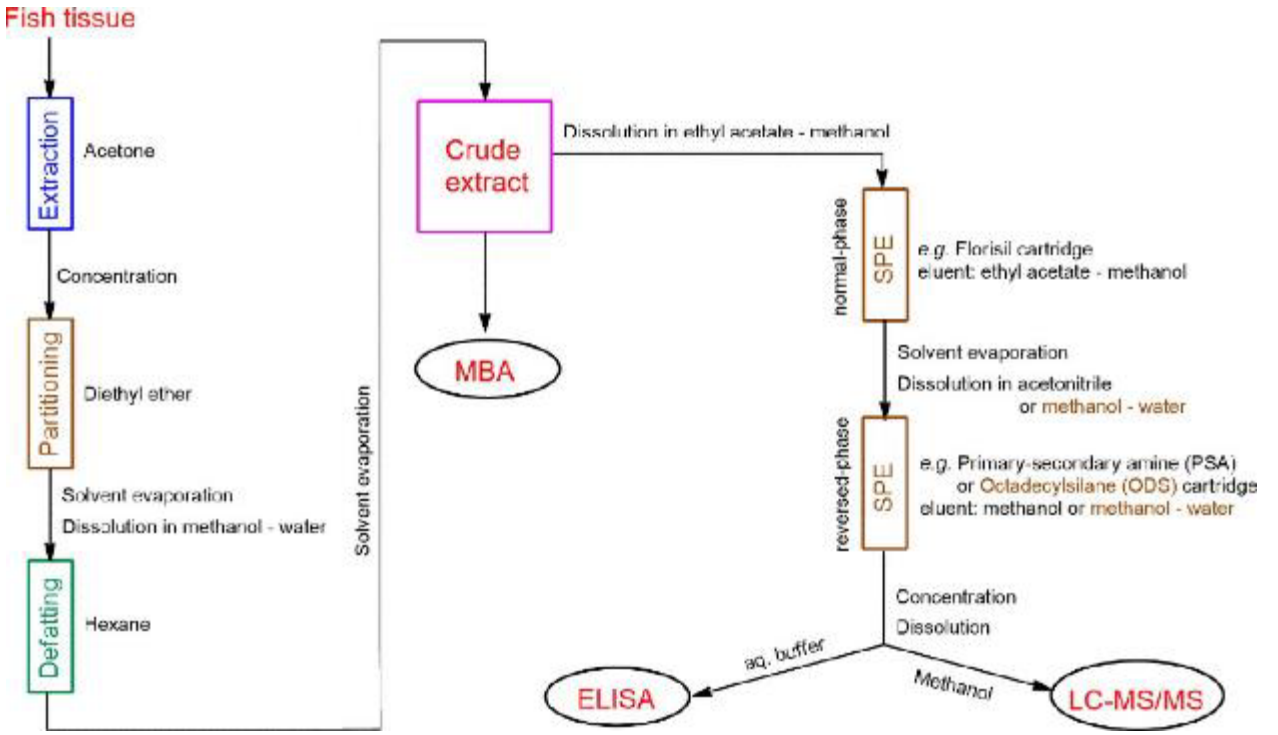


Figure 3. Diagram depicting the complex series of steps required for the extraction of CTX from fish flesh with subsequent application of several different solvents. Depicted here for samples to be tested on the mouse bioassay (MBA), enzyme linked immunosorbent assay (ELISA) or liquid chromatography coupled to mass spectrometry analysis (LC-MS/MS). Note that current extraction protocols for assays other than the MBA require solid phase extraction steps (SPE). Adopted from Pasinszki et al. (2020).

8.7 CTX detection and quantification

The detection and quantification of CTXs in fish tissues is uniquely challenging due to their high potency, occurrence at low concentrations, complex structure and the scarcity of analytical standards. There currently is no official, reference, or routine method to detect CTXs and no international agreements on specifications or standards to meet, either for foodstuffs or environmental screening (WHO, 2020). Early work utilised a range of different bioassays to detect CTXs, including the use of mongooses (Hokama et al., 1977), chickens (Vernoux et al., 1985), cats (Larson & Rothman, 1967), brine shrimp (Granade et al., 1976), Diptera larvae (Labrousse & Matile, 1996), mosquitoes (Bagnis et al., 1979) and mouse bioassay (MBA). Ethical, as well as practical considerations led to the later development of much more refined cell line or functional assays, such as the N2a cell line or receptor binding assay (RBA), that capitalise on the affinity of CTXs to site 5 of voltage gated sodium channels. Several different protocols for these assays have emerged over time and efforts are currently underway to standardise and fine-tune their application (e.g. Viallon et al., 2020). Furthermore, excellent reviews on the historic and current development of CTX detection methods are available (e.g. Pasinszki et al., 2020, see Figure 4 for historic timeline of CTX method development). These analytical techniques are briefly described below to provide context in relation to the time and infrastructure required to conduct these tests. CTX analytical techniques can be divided into screening (detection of broad spectrum CTX-like activity) and quantitative analysis (detection & quantification of specific CTXs).

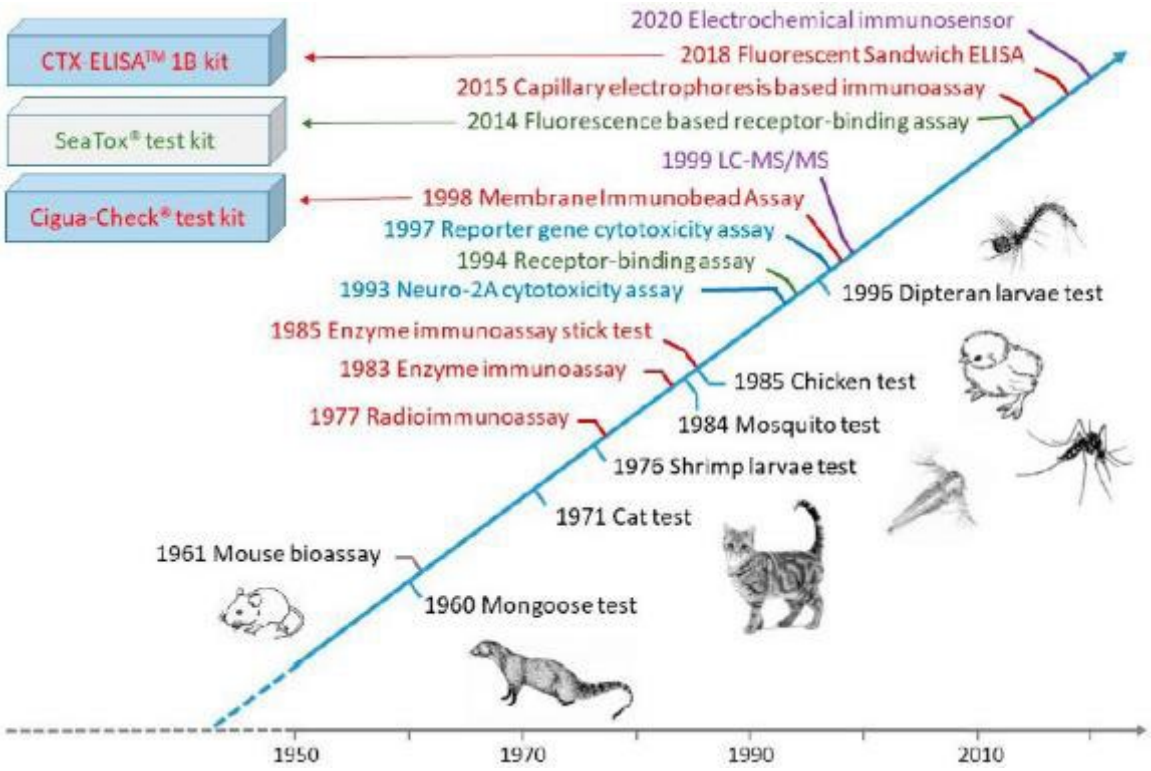


Figure 4. Timeline of the development of CTX analytical techniques (from Pasinszki et al. 2020). The most applicable assays are divided into CTX screening and quantification techniques and summarised in more detail below.

8.8 CTX screening techniques

Screening techniques are designed to detect the presence/absence of CTX-like activity. These assays are mostly cell-based or functional assays that report comprehensive toxicity (i.e. total toxicity of all CTX analogues present). While the results can often be expressed as CTX-like activity equivalents (if a CTX standard is available), these assays do not give information on what type of CTX analogues may be present. Depending upon the assay type, toxicity or biological activity of other naturally co-occurring compounds may be indistinguishable from the CTX signal. The most widely employed CTX screening techniques are briefly described below and key attributes listed in Table 1 for ease of comparison.

8.8.1 Mouse bioassay (MBA)

The MBA is the only animal assay still in use and is based on observing the time of death of mice that have been exposed to suspect samples via either intraperitoneal injection or sometimes feeding of suspected CTX containing samples (EFSA, 2010). This assay detects total toxicity and does not give detailed information on the types of CTXs in the sample, their concentrations or other toxicants potentially present. Originally widely used for the detection of marine biotoxins, the mouse bioassay is now being phased out for ethical reasons and preference given to LC-MS quantification of specific marine biotoxins. While the MBA does not require specialised analytical machines, it is expensive due to the requirement for animal rearing and testing facilities. CTX are highly toxic to mice via either intraperitoneal or oral delivery (Lewis et al., 1991) and CP caused by CTX may be overestimated when using the MBA (Pasinszki et al., 2020).

8.8.2 N2a assay

This assay is based on the sodium channel activating activity of CTXs. Mouse brain cells rich in sodium channels (N2a cells) are grown in 96-well microplates and incubated for a 24 h period, after which they are

sensitised to CTX-like activity through the addition of a series of chemicals. Upon a 24 h exposure to a suspect sample, any CTX present will activate the sodium channels in the neuroblastoma cells, causing an influx of sodium ions that leads to impaired cell viability. After 24 h of exposure, the viability of the remaining cells is measured with a fluorescent stain that when compared to a non-toxic control gives an estimate of CTX-like activity. This assay is not specific to CTX, as it will also detect activity of other sodium channel activating substances (e.g. brevetoxin) or compounds with general cytotoxic activity (e.g. maitotoxins). The N2a assay can detect total CTX-like activity within the range known to cause illness, but requires a prolonged incubation time and constant maintenance of the cell line assay.

8.8.3 Fluorescence Imaging Plate Reader assay (FLIPR)

This cell line assay is based on the use of human brain tissue cells that express several sodium channel types in their cell membranes (cell line SH-SY5Y). Treatment of this cell line with veratridine prior to testing sensitises it to CTXs. The cell line is preloaded with a fluorescent dye that when the cells are exposed to CTXs, emits a strong fluorescent signal. This increase in fluorescence can be quantified with a fluorescence imaging plate reader (Lewis et al., 2016). Like the N2a cell based assay, the FLIPR assay also requires plating of cells 24 h prior to testing, but the time of analysis is considerably shorter (~35 min compared to 24 h for the N2a). Major drawbacks are the need for specialised, expensive machinery (FLIPR), cell line maintenance and the incubation time prior to analysis.

8.8.4 Receptor binding assay (RBA)

Similar to the cell line based N2a and FLIPR assays, the RBA is based on the affinity of CTXs and brevetoxins to bind to sodium channels. Using sodium channel rich preparations of mouse brain tissues (synaptosomes), the assay measures the competitive binding of radioactively labelled brevetoxin (control) and CTXs (sample). The amount of labelled brevetoxin that can bind to the sodium channel is proportional to the concentration of ciguatoxin(s) in the sample. If no CTX is present, all binding sites will be occupied with radioactively labelled brevetoxin. As the concentration of CTXs in the sample increases, the bound amount of radioactive brevetoxin will decrease, as CTX competes for the identical binding sites. By comparing the reduction in the radioactive signal between the non-toxic control and sample, the CTX-like activity can be quantified. However, as for the N2a, total toxicity is measured and no CTX analogue specific information can be obtained.

A modified version of this assay using fluorescently instead of radioactively labelled brevetoxin has been developed through a collaboration between US Universities, Government Departments and the Institut Louis Malardé (abbreviated as fRBA, Hardison et al., 2016). Using fluorescent labelling eliminates the need for specialised facilities to work with radioactive materials. A commercial version of this assay is available from SeaTox Research Inc. (Wilmington, USA) and allows for the simultaneous detection of CTX in 48 samples within 3 hours. However, this time does not take into account the time required to prepare the toxin extracts from fish tissues. Current use of the fRBA method requires the clean-up of samples prior to analysis using SPE cartridges (Hardison et al., 2016; Litaker et al., 2014), but efforts by French Polynesian researchers are currently underway to fine-tune the extraction technique (pers. com. Mireille Chinain, Institut Louis Malardé).

8.9 CTX Quantification of specific CTX analogues

While the above-described screening methods focus on detecting CTX-like activity, quantitative analysis provides specific detail on the type and concentration of individual CTX analogues if toxin standards are available.

8.9.1 Enzyme linked immunosorbent assay (ELISA)

In principle, these assays are based on the development of an antibody that is specific to a certain part of the CTX molecule. The antibody is radioactively, fluorescently or enzymatically labelled and can be quantified

when bound to the toxin molecule. The specificity of the antibody to the toxin molecule plays an important role. While some may only bind to specific CTXs, others may not be as specific and cross-react with different CTXs or similar chemical structures on related compounds. A series of ELISA based assays for the detection and quantification of different CTX have been developed over the years, including two commercial rapid test kits (Cigua-Check® and Ciguatect™). Both of these test kits are no longer available, largely due to concerns about their sensitivity and specificity, as well as interpretation of results (Bienfang et al., 2011; Pasinszki et al., 2020). However, a new sandwich hybridisation ELISA has recently been made commercially available. This assay uses highly specific antibodies and can selectively detect trace amounts (<1 pg/mL) of P-CTX-1 and 54-deoxyCTX-1B (Tsumuraya & Hirama, 2019), but not other CTXs that may potentially be present. The fish tissue sample extraction technique given in the manufacturer's guidelines does not require the additional SPE clean-up steps, although the limits of detection supplied for this assay are based on Tsumuraya and Hirama's work that employed two additional SPE clean-up steps (Tsumuraya & Hirama, 2019). While the limit of detection for crude tissue extracts (no SPE) remains unknown for this assay (a major drawback), an earlier, less sensitive ELISA was able to be conducted on crude extracts without apparent protein or lipid interference (Campora et al., 2008b). As per the manufacturer's protocol, the assay is limited in that only six samples can be run per test kit when quantifying CTX concentrations.

Preliminary tests with this commercial kit have shown strong fluorescence responses in the presence of CTX when compared to non-toxic controls (pers. com. Sam Murray, Cawthron Institute). This offers the possibility of running the assay in a qualitative screening mode (presence/absence of CTXs), which would dramatically increase the number of samples that can be run on a single plate (up to 48 samples), as the dilution series required for the quantification mode would no longer be required. A single assay kit currently costs around $1250 excluding international hazardous material shipping from Japan.

8.9.2 Electrochemical biosensor

An electrochemical biosensor using identical antibodies as the commercial ELISA has recently been introduced by Leonardo et al. (2020). While the sensor can detect P-CTX-1B and 54-deoxyCTX-1B down to 0.01 µg/kg (FDA guidance level), it still requires sample extraction and is in the early stages of development (i.e. not commercially available).

8.9.3 Liquid chromatographic methods (LC-MS and LC-HRMS)

Liquid chromatography coupled to mass spectrometry (LC-MS) or high-resolution mass spectrometry (LC-HRMS) has replaced the mouse bioassay for the detection and quantification of several marine biotoxins. While these techniques require highly skilled operators and expensive infrastructure, they allow for the separation, identification and quantification of individual CTXs, provided that specific CTX standards are available. Rather than reporting total toxicity, these approaches detect and quantify CTXs (and metabolites) based on their chemical properties. Like all other CTX analytical techniques listed above, these methods are subject to matrix interferences and require clean-up of samples prior to analysis (including SPE steps).

LC-MS techniques to detect P-CTX-1B in Spanish Mackerel have been set up at the Sydney Institute for Marine Sciences (FRDC project no. 2014-035) to measure P-CTX-1B in Spanish Mackerel flesh and liver samples collected during the most comprehensive screening of NSW fish to date (Kohli et al., 2016).

Table 1. Available methods for the detection and quantification of CTXs in fish tissues summarising key characteristics. This table has been modified from the proceedings of the 2018 WHO expert meeting on ciguatera poisoning to summarise key characteristics of CTX analytical techniques. The limit of detection (LOD and the limit of quantification (LOQ) are also provided.

Assay	Type of analysis	Pros	Cons	Time*	LOD	LOQ	Infrastructure required
Receptor binding assay (RBA) - Radioisotope	Screening	Detects total toxicity. High throughput. Highly sensitive.	Does not distinguish between types of CTX. Requires radioactive materials.	3-5 h	P-CTX3C: 0.115 ng/g	P-CTX3C: 0.31-0.33 ng/g	Clean work environment (radioactive material), plate reader, cell line culturing facilities
Receptor binding assay (RBA) - Fluorophore	Screening	Same as RBA above, but does not require radioactive materials. Commercial kit available. Highly sensitive.	Does not distinguish between types of CTX.	3 h	P-CTX3C: 0.075 ng/g	P-CTX3C: 0.1 ng/g	Clean work environment, plate reader, microplate filter manifold, freezer.
Mouse bioassay (MBA)	Screening	Detects total toxicity.	Does not distinguish between types of CTX or between CTXs and other toxins. Ethically questionable & requires expensive animal housing infrastructure. Also requires supply of mice at specific age.	24 h	P-CTX1B LD_{50}: 0.25 ng/g		Specialist animal facilities and training required.
N2a cell-based assay	Screening	Detects total toxicity. High throughput. Highly sensitive.	Does not distinguish between types of CTX or between CTXs and other toxins. Very sensitive to sample matrix effects (e.g. protein & lipid content). Cells are required to be plated 24 h before analysis.	24 h (+24 h)	P-CTX-1B: 0.0096-0.17 ng/g P-CTX3C: 0.02 ng/g	P-CTX-1B: 0.4-17 pg/g	Sterile work environment, cell line incubator, plate reader, temperature controlled cabinets.
FLIPR cell-based assay**	Screening	Detects total toxicity. High throughput. Highly sensitive.	Does not distinguish between types of CTX or between CTXs and other toxins. Cells required to be plated 24 h before analysis. Has not yet been tested in fish.	35 min (+24 h)	P-CTX-1B: 0.025 ng/ml		As N2a above, plus a specialised fluorescence imaging plate reader

Assay	Type of analysis	Pros	Cons	Time*	LOD	LOQ	Infrastructure required
ELISA	Quantitative or qualitative	Detects specific toxicity of P-CTX-1B (and 54-deoxy CTX1B) or P-CTX3C (and 51-hydroxyCTX3C). High throughput (96-wells). Can be used in qualitative mode to screen multiple samples at ones. Commercial kit available. Highly sensitive and specific.	Specific to 3 Pacific CTX analogues only (commercial kit only tests for P-CTX-1B and 54-deoxyCTX1B. Expensive in quantitative mode (can only run 6 samples).	2 h	P-CTX-1B: 0.16 pg/g P-CTX3C: 0.09 pg/g 51-hydroxy-CTX3C: 0.1 pg/g 54-deoxy-CTX1B: 0.11 pg/g	P-CTX-1B: 0.49 pg/g P-CTX3C: 0.27 pg/g 51-hydroxy-CTX3C: 0.3 pg/g 54-deoxy-CTX1B: 0.32 pg/g	Clean work room, plate reader, fridge
LC-MS/MS & LC-HRMS	Quantitative/ confirmatory	Highly CTX specific and allows for quantification of individual analogues. Detection of unknown toxins. Highly sensitive.	Requires expensive, highly specific machinery, highly trained operator and additional sample preparation & clean-up. Reliant on availability of toxin standards.	15-30 min	P-CTXs 0.01-0.02 µg/kg for individual analogues	P-CTX3C: 0.01-0.05 µg/kg for individual analogues	Clean work environment, expensive instrumentation (>$700,000), highly skilled operator

* Times given here are for analytical time only and do not include time required for sample preparation/extraction of CTX from fish tissue (depending on technique, requires ~6.5 h to multiple days).

** Not tested in fish flesh to date, but successfully applied for detection of CTX in microalgal culture extracts by Lewis et al. (2016).

8.10 Incorporating CTX analytical methods into risk management

Three major approaches to incorporate CTX analytical methods into risk management have been identified:

i. Random screening of marketed/imported fish

This approach is based on the random selection of fish at market to be tested for the presence of CTX. Additional criteria might be applied, such as targeting fish of certain size, species or origin. This approach is currently practiced in France, where official monitoring for CTX involves selecting samples from fish species listed as risky overseas using a random sampling grid. The selected fish are analysed via the MBA. A similar program operates in the United States of America, where the FDA randomly screens fish from areas identified to be of higher risk. In the USA, fish samples are first screened for CTX-like activity with the N2a assay and positive detections followed up with quantitative LC-MS. Due to an inability to detect specific CTXs and ethical considerations, the MBA is not considered an appropriate method for current research needs. A screening approach with alternative tests methods could potentially be applied to NSW Spanish Mackerel, but would require improved knowledge of the prevalence of CTX in this species to determine the level of screening required each fishing season.

ii. Size-specific screening of harvested fish

Certain fish species are considered to be of higher risk of carrying ciguatoxins at concentrations sufficient to cause illness. Rather than randomly selecting fish as described above, this approach requires the screening of all fish of a certain species that exceed a given size limit (Table A7). An example is the management plan for commercial fishing in the Canary Islands. Seven locally caught fish species are deemed to be at higher risk (based on local experience) and species-specific weight restrictions placed on them. Fish that exceed these weight limits can be presented to the local Fisheries Department, where a 300 g flesh sample is extracted to be tested on the N2a cell line assay (Sanchez-Henao et al., 2019). The fish are stored frozen in an authorised cold store until analytical results are available and only proceed to market if tested negative for CTX. This process has the advantage that the data collected during the monitoring program could then be used to model the risk of CP in specific fish species and identify and refine risk factor levels, including size of fish, season and fishing locations (as occurs in the Canary Island monitoring program: Sanchez-Henao et al., 2019). While freezing of fish whilst awaiting test results is not feasible for the fresh product-based NSW Spanish Mackerel industry, size-specific screening of fish would offer the opportunity to comply with SFM guidelines. However, to screen fish before market, a potential screening test would have to be rapid (<4.5 hours, see discussion below) and this management approach informed by improved fish size specific CTX prevalence data.

iii. Environmental monitoring

Environmental monitoring of CTX aims to identify whether certain fishing locations are at risk of harbouring ciguateric fish. Typically, this involves yearly monitoring of CTX in fish known to bioaccumulate CTX, with a focus on fish species that are true to their location, i.e. non-migratory. This approach has been employed in the Cook Islands and French Polynesia to identify locations that are more prone to harbouring ciguateric fish (e.g. Chinain et al., 2010). Current evidence suggests that CTX concentrations can vary widely between individual fish at the same location and fish a few kilometres away may not be contaminated. The small geographical scale at which fish would need to be sampled to inform food safety management would make it difficult (and expensive) to implement this environmental monitoring across multiple fish species. This approach is complicated for migratory fish, such as Spanish Mackerel, which may frequent several of these zones during their migration from QLD to NSW. However, monitoring a sentinel fish species known to rapidly respond to ciguateric activity in a given region may serve as an indicator of increased risk of CTX contamination in specific localities and/or across different fishing seasons. A variation of this approach is practiced in Australia, where fish are not accepted to market if they originate from a specific location considered to be of higher ciguatera risk, such as Platypus Bay in QLD and the Gove Peninsula in the NT. There is currently no CTX monitoring program in place at these locations that operates on a consistent basis.

This is partly due to the limitations of the sampling effort involved and the historic limitations of ciguatoxin detection techniques (e.g. time required for ciguatoxin extraction).

8.11 Discussion

The detection and quantification of CTXs has significantly advanced in the last decade, but some of the historic challenges have remained: no validated reference techniques currently exist, CTX standards remain largely inaccessible, lengthy extraction protocols must be adapted to individual fish tissues and sample purity requirements differ between analytical techniques. The lack of commercially available CTX standards in particular limits the extent to which confirmatory analysis (e.g. LC-MS) can be performed. This is where cell line based and functional CTX assays that detect total sample toxicity (all CTXs and potentially other toxicants) are useful to pre-screen samples for the presence of CTX-like toxicity, as they do not require CTX standards. While following up any positive screening detections with confirmatory analysis would be beneficial to gain information on individual CTX analogues and their concentrations (providing CTX standards are available), a positive screen test result may be sufficient to reject the fish from a food safety perspective. This is providing that the chosen screening technique has been shown to reliably detect the presence/absence of CTX in a specific fish tissue without giving unacceptable numbers of false positives (positive result despite no CTX present, resulting in CTX free fish wrongly rejected) or worse, false negatives (negative result despite CTX being present, resulting in ciguateric fish entering market).

While monitoring of CTX for food safety management purposes is currently not practised in many countries due to the lack of reference techniques and standards, screening assays detecting total toxicity have been successfully employed to specifically target certain fish sizes that would otherwise be considered too risky for human consumption (e.g. in the Canary Islands, Sanchez-Henao et al., 2019). While larger, predatory fish were generally considered to have a higher risk of carrying CTXs, the relationship between fish size and CTX concentration appears to be fish species specific (Gaboriau et al., 2014). Some fish species, such as Red Bass (*Lutjanus bohar*), show a good relationship between increasing body size and CTX concentration, whereas the majority of other fish species do not (Gaboriau et al., 2014). Previous screening of NSW Spanish Mackerel detected no clear relationship between fish size and the presence/absence of CTX in either fish flesh or liver tissues (71 fish tested over one fishing season, Kohli et al., 2016).

Of the six NSW fish that tested positive for P-CTX-1B, toxin concentrations in the liver were approximately six times higher than in the flesh (Kohli et al., 2016). Overseas investigations have reported similar findings of higher CTX concentrations in fish livers of other species, such as moray eel (Chan et al., 2011; Yasumoto & Scheuer, 1969). This indicates that the fish liver could be a suitable target tissue for CTX screening analysis, as higher CTX concentrations may offset the detection thresholds of certain extraction and analytical techniques to provide a higher probability of detecting ciguateric fish. It should be noted, that the currently available data on CTX in Spanish Mackerel is limited in that it covers 71 fish from NSW from a single fishing season (2015), allowing comparison of fish liver vs. flesh CTX concentrations for only six CTX positive samples (Kohli et al., 2016). Additional data on the prevalence of CTX in NSW fish is required to generate higher confidence in relative CTX prevalence estimates between fish tissues.

Several different CTX screening techniques are currently available, all of which require sample extraction and remain unvalidated for use in Spanish Mackerel flesh or liver samples. Of these techniques, perhaps the most promising are the commercially available fluorescently labelled receptor binding assay (fRBA) and the Japanese ELISA test kit. A major advantage of these assays over the cell line based N2a and FLIPR methods is that they do not require constant cell line maintenance or plating of cells 24 hours prior to analysis. The fRBA test kit can be kept in the freezer (shelf life of 6 months) and the ELISA stored in the fridge until analysis. The ELISA and fRBA assay reagents are supplied individually, so that it is not required to run all 96-wells in a microplate at once, providing flexibility if shorter sample turnaround is required and fewer samples are to be analysed on a given day. Another characteristic that makes these two screening assays attractive, is their short analytical time of 2-3 hours.

However, the analytical time does not account for the time required for CTX extraction. The most recent work employing these two assays to detect CTX in fish flesh have employed time consuming SPE clean-up steps during sample preparation (Hardison et al., 2018; Hardison et al., 2016). While this is a common requirement for most analytical techniques, previous work on a sandwich ELISA test successfully analysed crude Amberjack and Grouper extracts without apparent sample matrix interference (no SPE, Campora et al., 2008b). The extent of potential matrix interference is not only dependent upon the number and type of extraction steps, but also the tissue matrix itself (e.g. Spanish Mackerel flesh vs. oily liver vs. blood). Whether CTX in crude Spanish Mackerel tissue extracts can be detected at levels low enough to confidently screen fish before market with these techniques, will need to be investigated.

An attractive alternative to the long extraction procedures required to analyse CTX in fish flesh or liver samples, is the use of fish blood. To date, CTXs have been successfully detected in grouper, barracuda, snapper and moray eel blood samples, but the relationship between CTX concentrations in fish liver, blood and flesh for these fish requires further research (Dechraoui et al., 2007; Mak et al., 2013; O'Toole et al., 2012). A key consideration is the time period over which CTXs stay in the blood after individual fish have consumed CTX containing prey. Spanish Mackerel are thought to pick up CTXs in QLD waters before migrating down the coast into NSW. There is a risk that the time taken to migrate may reduce CTX levels in the blood to below the detection thresholds of current analytical techniques, while significant CTX concentrations remain bound in the fattier liver and/or flesh tissues. However, the much simpler fish blood matrix promises considerably reduced extraction times and should be looked at in concert with flesh and liver tissues.

The ELISA has the lowest CTX detection limit of all currently available analytical techniques, almost 2 orders of magnitude lower than the FDA guidance level (Tsumuraya & Hirama, 2019). This low limit of detection will likely prove advantageous for the detection of CTXs in the more complex matrix of crude tissue extracts, but the extent to which impurities in these preparations may cause false positives through high background fluorescence, remains to be determined. The high specificity of the ELISA antibodies to P-CTX-1B and 54-deoxyCTX-1 means that only these two analogues will be detected. Unlike the ELISA, the fRBA gives a comprehensive picture of total toxicity, including other types of CTX, such as P-52-EPI-54-DEOXY-CTX-1B (FORMERLY KNOWN AS CTX-2) and -3. While P-CTX-1B is the most potent Pacific ciguatoxin detected in Australia and other P-CTXs have not been reported in the absence of P-CTX-1B in fish implicated in human poisonings (Farrell et al., 2016a; Hamilton et al., 2010, and QLD Forensic & Scientific Services data), additional data on the prevalence of P-CTXs in Spanish Mackerel will provide higher confidence in focusing analysis solely on P-CTX-1B.

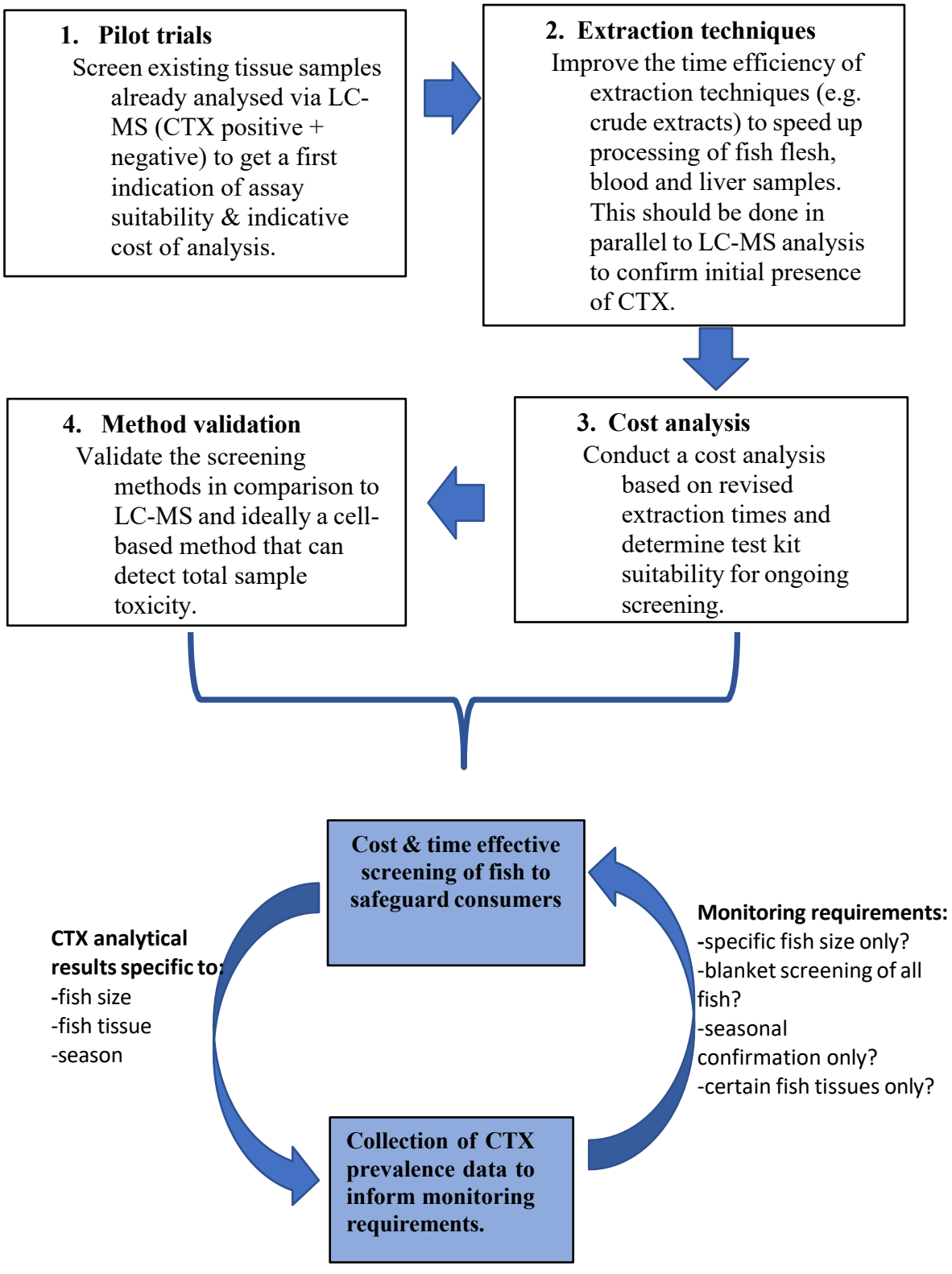
To investigate the suitability of the ELISA or fRBA test kits for the screening of fish, pilot trials will be required. Ideally, these trials would be run in tandem with other detection techniques, such as LC-MS or the N2a assay. If satisfactory detection is achieved, the next phase should target the refinement of extraction techniques to reduce time and cost of analysis. Of particular interest is the distribution of CTXs between flesh, blood and liver tissues. While the flesh is the main part of the fish that is consumed, the liver may harbour higher CTX concentrations that may be more readily detected. Screening the blood is of interest, as it presents a much simpler matrix that allows for much simpler and shorter extraction procedures than those required to extract tightly bound CTXs from fish flesh. However, more data on the relative CTX concentrations between fish tissues is required to confidently infer the presence/absence of CTX in fish flesh based on toxin concentrations in either liver or blood.

However, when combining current sample preparation time (includes SPE extraction steps) and approximate assay costs (~$1250 per test kit), blanket screening all Spanish Mackerel before going to market would not be cost effective when considering the market price of Spanish Mackerel (current retail value ~ $30/kg). This is largely due to the time required for sample extraction following currently employed LC-MS protocols (8 h to extract 10 samples). This in turn limits the number of samples that can be analysed in a given day and increases the overall cost per sample to approximately $70 for the fRBA and $95 for the ELISA when focusing on “quick” turnaround (i.e. extraction and analysis of 10 samples, which takes ~1.5 days to result). Freezing of fish would allow for a full complement of 48 samples to be run at once, significantly improving the cost-effectiveness of analytical labour. However, this is not an option for the Spanish Mackerel industry,

which is entirely based on high quality, fresh product (pers. comm. Tricia Beatty, NSW PFA). Consequently, the Spanish Mackerel supply chain leaves little time for analysis, let alone sample extraction. The only time window where fish are at a centralised location, exists between the fish arriving at the Sydney Fish Market (1- 2 am) and the start of the auction (5:30 am). This leaves 3.5-4.5 hours for unloading of fish, labelling (for later identification should CTX be detected), sample collection, extraction, analysis and reporting. Delaying the introduction of Spanish Mackerel to the auction floor could potentially provide additional time (2 h). Taking into account the time required for analysis 2-3 hours and sample management, extraction techniques (currently 6.5 hours) would have to be much refined to efficiently and reliably extract CTX within ~2 hours. It therefore remains questionable whether the fRBA and ELISA test kits will ever be an effective option to blanket screen all Spanish Mackerel before market as part of an ongoing monitoring regime.

Instead, a more viable approach appears to be the fine-tuning of these two assays and sample extraction techniques to gather vital information on the occurrence of CTX in NSW Spanish Mackerel on a cost-effective basis. This information could then in turn be used to determine the future needs for CTX monitoring and help to potentially review the Sydney Fish Market ciguatera guidelines regarding the exclusion size limit of Spanish Mackerel. This would have to be supported by improved CTX prevalence data.

Should either of the fRBA or ELISA prove suitable for the detection of CTX in Spanish Mackerel tissue, their use for food safety management will need to be properly validated. In the absence of ciguatoxin standards, this requires the use of several fish samples identified to be CTX positive by multiple techniques, including confirmatory LC-MS analysis. However, the complete validation of either of these two test kits and their associated improved extraction techniques will be outside the scope of the present project and would need to be conducted separately, (should pilot trials prove successful). Once validated, both the ELISA and fRBA tests could be run with standard fluorescent plate readers available in most analytical laboratories. It is important to note that the progression from pilot trials to screening method validation is a step-by-step process that directly adds to the understanding of CTX prevalence in Spanish Mackerel. This data will be key to inform the future needs for monitoring. The approach from pilot trials to test kit validation and improved monitoring is summarised in a flow chart. The series of sequential steps required to test the suitability of the fRBA and the ELISA test kits to detect CTX in Spanish Mackerel flesh, blood and liver tissues before either of these techniques may be employed for the collection of CTX prevalence data:



8.12 Conclusions

The NSW Spanish Mackerel fishery is a small fishery with a short supply chain that sells directly fresh to market the day after catch. This limits the options of installing a potential CTX control point to a 4.5-6.5 h window between fish arriving at the Sydney Fish Market late at night (1-2 am) and the start of the auction early in the morning. Current company guidelines prohibit the sale of Spanish Mackerel exceeding 10 kg (or 8

kg headed & gutted), but larger fish (>10 kg) are more prevalent and lucrative in terms of catch & effort (currently discarded because of perceived CP risk). This provides a strong economic incentive to either implement a rapid CTX testing program to screen fish before market and/or revise the Sydney Fish Market guidelines based on an improved understanding of the prevalence of CTX in NSW Spanish Mackerel.

Of the currently available CTX screening techniques, the commercially available fRBA and ELISA test kits offer the best promise of quick, high throughput analysis, but their suitability for detecting CTXs in Spanish Mackerel blood, flesh or liver tissues remains to be investigated. Of particular interest is their potential use to detect CTXs in crude tissue extracts without lengthy SPE extraction steps. Based on the current NSW fishery supply chains and the time estimates of current extraction techniques, these test kits will not be suitable for blanket screening of all Spanish Mackerel going to market. Instead, their use as screening tools to collect additional CTX prevalence data should be investigated to revise current management guidelines and inform future monitoring efforts.

9 Spanish Mackerel in Australia

The following is a summary of information on the distribution, stocks, biology, life history, migration, and fisheries in the Australian east coast stock of Spanish Mackerel. A particular focus is the current understanding of the southern migration of Spanish Mackerel into NSW waters and the influence of environmental variables on this, including projected changes under future climate change scenarios. The summary information presented on distribution, stocks, biology and life history is necessary background to the dynamics of the migration and the associated fisheries. It is not intended to be an exhaustive review of these topics, but it covers the main information sources from the published international peer-reviewed literature and the grey literature.

9.1 Distribution

Narrow-barred Spanish Mackerel (*Scomberomorus commerson*) in Australia occur throughout northern tropical and sub-tropical waters to about 30°S. They are occasionally recorded as far south as Geographe Bay (Western Australia) and St Helens (Tasmania) (Tobin & Mapleston, 2004) (Figure 5).

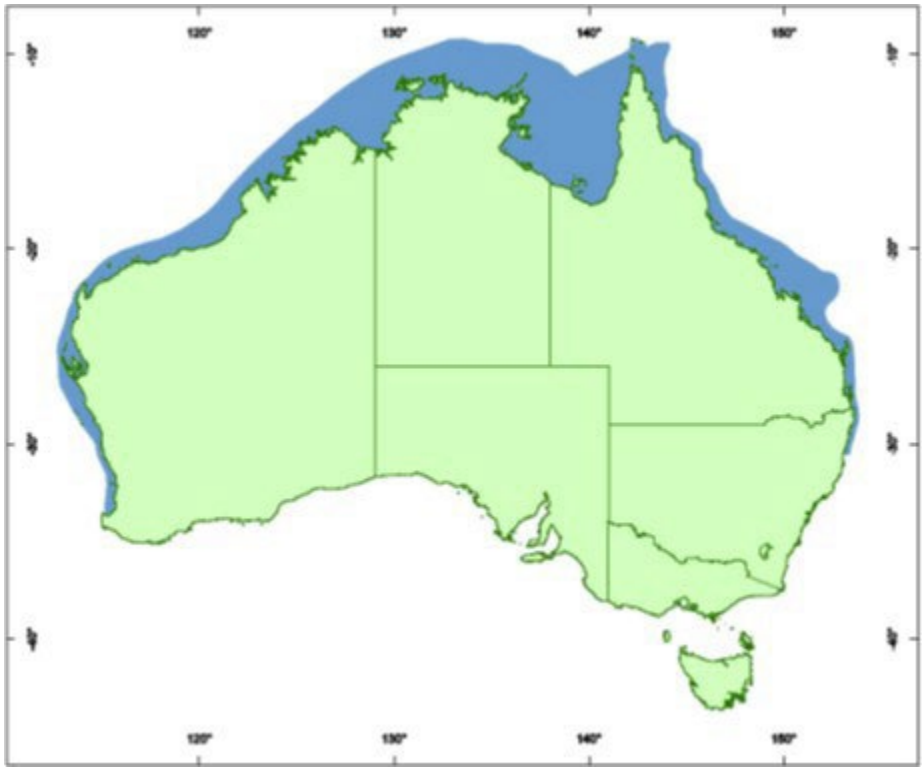


Figure 5. Australian distribution of Spanish Mackerel. (Welch et al., 2014)

Environmental Variables Underlying the Current Distribution of Spanish Mackerel and Projected Changes in Distribution due to Climate Change. Champion et al. (2021) found the distribution of Spanish Mackerel on the east coast of Australia was related to three oceanographic variables: sea surface temperature (SST; 0.05° spatial resolution), sea level anomaly (SLA; 0.25° spatial resolution), and chlorophyll a concentration (CHL; 0.04° spatial resolution) as summarised below in Figure 6.

Table 1. Summary of the optimal oceanography-only (∗) and topography-inclusive (◎) SDMs for each study species. Smoothing factors applied to environmental covariates are indicated by "s." AUC and TSS model evaluation statistics are derived from temporally explicit sixfold cross-validation, where oceanographic-only and topographic-inclusive models for each species were trained and tested on subsets of data from the periods 1998–2008 and 2009–2018. SST, sea surface temperature; EKE, eddy kinetic energy; CHL, chlorophyll *a* concentration; SLA, sea level anomaly; TOPO, coefficient of topographic variation.

Model	ΔAIC	Mean AUC (± SD)	Mean TSS (± SD)
Spanish mackerel (*Scomberomorus commerson*)			
∗s(SST) + s(CHL) + s(SLA) + (1|Year)	114.6	0.728 ± 0.009	0.545 ± 0.008

Figure 6. Summary of full models for each species and nested alternatives assessed using AIC informed model selection procedure on covariate combinations of decreasing complexity (Champion et al., 2021).

East coast Australian ocean temperatures have risen 4x faster than the global average over the last 60 years. (Ridgway 2007; Suthers et al 2011). This is being driven by strengthening of the East Australian Current due to increased wind stress over the South Pacific. This is leading to southward range shifts in many marine species. When climate change-driven projected alterations in the important oceanographic variables are modelled, the core oceanographic habitat of Spanish Mackerel is projected to move poleward at the rate of 278.6 km per decade (95% CI 223.6–333.7 km per decade), the maximum rate for the group of species studied (Champion et al 2021). The authors concluded that "fishing opportunity off south-eastern Australia is likely to be most rapidly increasing for Spanish Mackerel, followed by spotted mackerel, bonito and dolphinfish" (p 10). These changes in the distribution of fishes will have implications for the associated commercial and recreational fisheries, including increased fishing opportunities for affected species. Champion & Coleman (2021) subsequently refined their projections by incorporating the influence of habitat topographic complexity, which is a measure of the physical complexity of the seabed. They found that their explanatory model for the distribution of Spanish Mackerel was improved when topographic complexity was added (Figure 7):

Table 1. Summary of the optimal oceanography-only (∗) and topography-inclusive (◎) SDMs for each study species. Smoothing factors applied to environmental covariates are indicated by "s." AUC and TSS model evaluation statistics are derived from temporally explicit sixfold cross-validation, where oceanographic-only and topographic-inclusive models for each species were trained and tested on subsets of data from the periods 1998–2008 and 2009–2018. SST, sea surface temperature; EKE, eddy kinetic energy; CHL, chlorophyll *a* concentration; SLA, sea level anomaly; TOPO, coefficient of topographic variation.

Model	ΔAIC	Mean AUC (± SD)	Mean TSS (± SD)
Spanish mackerel (*Scomberomorus commerson*)			
∗s(SST) + s(CHL) + s(SLA) + (1|Year)	114.6	0.728 ± 0.009	0.545 ± 0.008
◎ s(SST) + s(CHL) + s(SLA) + s(TOPO) + (1|Year)	0	0.774 ± 0.010	0.602 ± 0.009

Figure 7. Summary of the influence of including habitat topographic complexity on projected changes in poleward distribution of Spanish Mackerel (Champion & Coleman 2021).

When topographic complexity was included in the modelled projections of rates of poleward range shifts by Spanish Mackerel under climate change it resulted in a 30.0% reduction in the rate of range shift (compared with the value in Champion et al., 2021) which is equivalent to a reduction of 94.4 (± 32.4 SE) km per decade.

9.2 Stock structure

There are 3 stocks of Spanish Mackerel in Australia: northern/western Australia, Torres Strait (which shows some similarities to both the northern/western and east coast stocks), and east coast Australia (Ovenden

& Street, 2007; Figure 8):

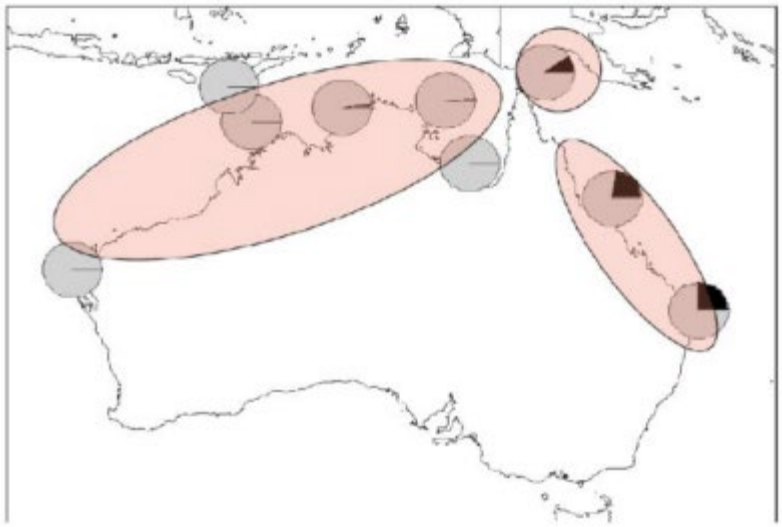


Figure 1 Australian Spanish mackerel genetic population structure (Ovenden and Street 2007).

Figure 8. Australian Spanish Mackerel genetic population structure (from Campbell et al. 2009))

The Spanish Mackerel caught in NSW are part of the east coast stock, which extends from Cape York to Newcastle (QLD Government 2022).

While the east coast stock appears to be genetically homogeneous, variation in parasite loads and otolith microchemistry suggest that the stock might be spatially structured into several metapopulations (based on non-migratory, resident individuals) at scales of 100-300 km that exhibit little/no mixing (Welch et al., 2014). There does not appear to be biological data at a sufficiently fine spatial scale to determine the boundaries of these metapopulations (but see the following section on regional differences in growth).

9.3 General Biology

Adults are highly mobile and epipelagic (i.e. living from the surface to about 100 m depth), schooling around reefs, shoals, headlands and current lines from coastal waters to the edge of the continental shelf. They are rarely found in waters greater than 100 m deep. Small juveniles (≤10 cm) occur in coastal creeks, estuaries, and mudflats during summer in North Queensland.

9.3.1 Growth and Age

Spanish Mackerel have a maximum longevity of 26 years, maximum length of 2400 mm (fork length FL), and become sexually mature at 2-4 years (800 mm FL) (SASF 2020). In the east coast stock females grow faster than males, reach a greater size (1550 mm, 35 kg) than males (1270 mm, 19 kg), and live longer (14 yr) than males (10 yr) (McPherson, 1992). Growth of Spanish Mackerel varies significantly among regions of the east coast of QLD (Ballagh et al., 2006):

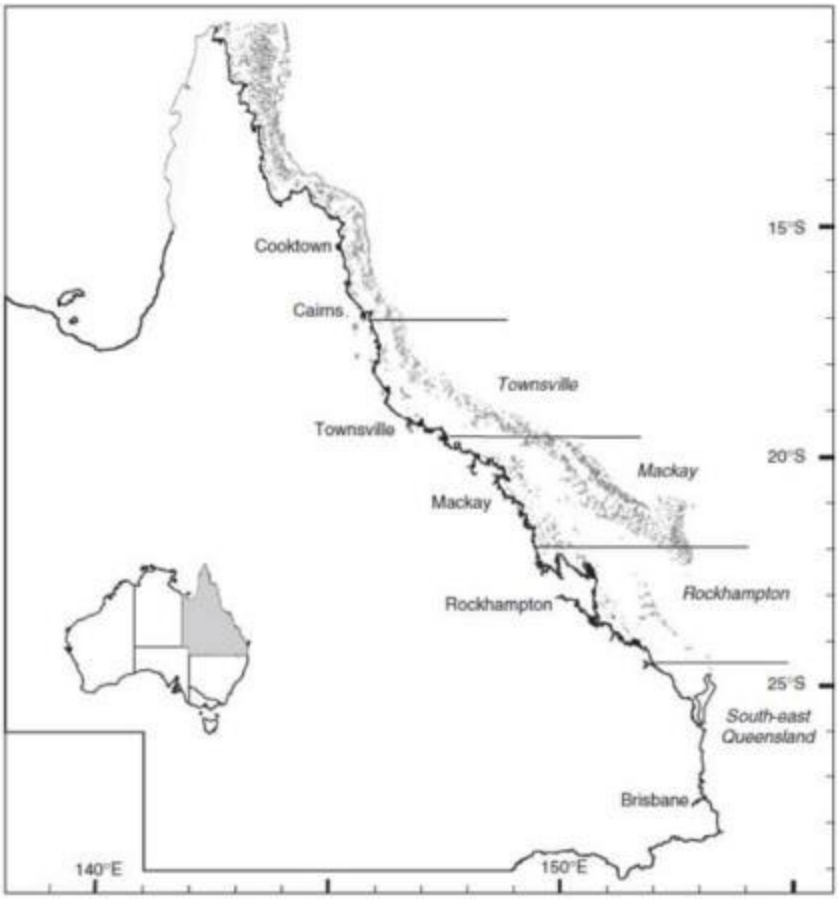


Figure 9. Locations of regions on the east coast of QLD studied to quantify growth variation of Spanish Mackerel (Ballagh et al., 2006).

Females and males had greater growth rates in the southern regions of Rockhampton and South- East Queensland than in the more northern regions of Townsville and Mackay (Figure 9). Females from Mackay reached a larger average maximum length than females from SE QLD. Male growth rate differed significantly among the regions, but only for males aged 1-2 years. Female growth rate differed significantly among the regions for ages 1-5 years. These observed differences in growth rates might be the result of a metapopulation stock structure, where that proportion of the population that does not migrate is influenced by differences in environmental conditions (or fishing pressure) among the regions.

9.4 Life History (East coast stock)

Every year, sexually mature Spanish Mackerel migrate during winter and spring to gather in large numbers as a prelude to mating in spawning aggregations that peak in October and November. In the 1970s these spawning aggregations formed on reefs between Townsville and Lizard Island but have more recently retracted to reefs between Townsville and Ingham (see following figure), most likely due to over-fishing reducing numbers of Spanish Mackerel (McPherson, 1981; McPherson, 2007; Welch et al., 2014). Spawning also occurs on reefs further south (between Gladstone and Bundaberg), for a shorter time (October-November), and does not involve large aggregations (McPherson 1981). No spawning has been recorded south of Fraser Island (Welch et al., 2002).

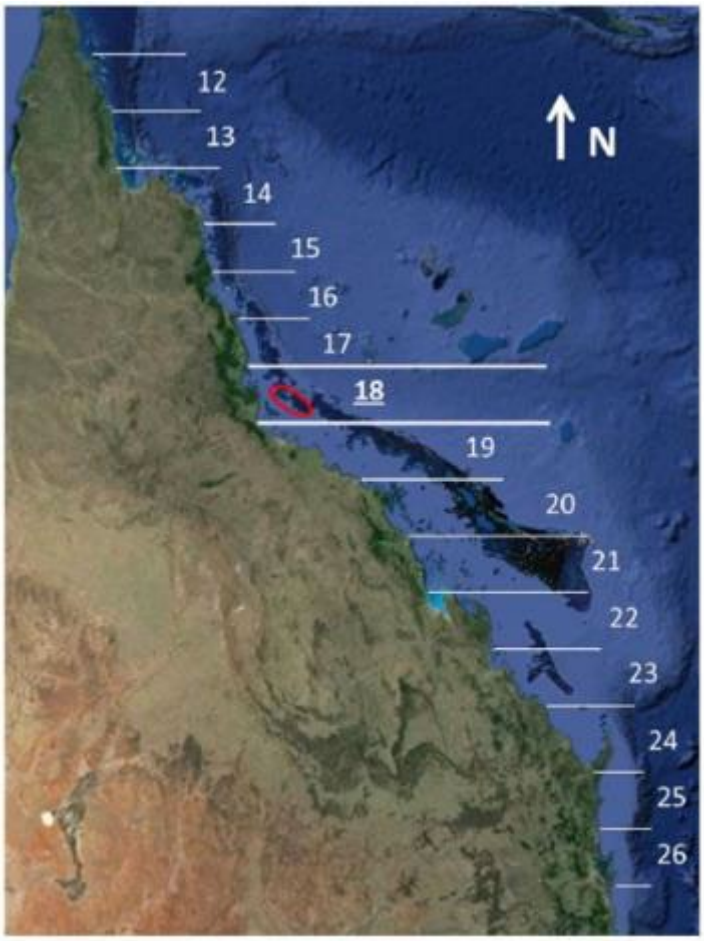


Figure 10. The QLD east coast with bands of latitude indicated. The historically important spawning aggregation fishery is largely confined within single band of latitude 18 degrees south. The red circle highlights the small group of inner shelf coral reefs where aggregations occur and are fished each spring (Tobin et al., 2014).

The timing of the spawning aggregation depends on sea temperature, and spawning generally occurs around the times of the new moons (Welch et al., 2014). The normally highly mobile Spanish Mackerel are very site-attached during the time they are present within the complex of spawning aggregation reefs. They aggregate at a particular reef and movement among the reefs of the spawning reef complex is rare. Once they leave that reef, they tend to leave the spawning reef complex (Tobin et al., 2014, Figure 10).

9.4.1 Early development

Spanish Mackerel have separate sexes with fertilization occurring externally in the open water following release of eggs and sperm. Larvae develop inside the fertilized eggs and hatch when they are about 2.5 mm long. The larval stage (spent drifting around in the open ocean) lasts 2-4 weeks during which they feed on other larval fishes and invertebrates (Welch et al., 2014).

At the end of the larval stage, the juvenile Spanish Mackerel make their way to inshore waters on the QLD east coast, to estuaries and intertidal sand flats in coastal bays, where they live and grow for around 6 months. They leave these inshore nurseries by May, moving offshore, when they are about 50 cm long. They

grow rapidly, reaching 65 cm FL by the end of their first year, and reach minimum legal size during their second year of life. Most are sexually mature by 2 years of age (Welch et al., 2014).

9.4.2 Post-spawning migrations

Post-spawning migrations of Spanish Mackerel from the spawning aggregation reefs occur between December and April when two types of movements occur by different segments of the stock: (1) long range migration (usually >700 nautical miles) into southern QLD and northern NSW waters; and (2) shorter range movements (<100 nautical miles). These are called, respectively, the migratory and resident components of the stock. The segregation into resident and migratory components occurred amongst fish that were two years and older (McPherson 2007).

The long-range migrations may be a way for larger fish to maintain favourable summer environmental or feeding conditions (McPherson 2007). The existence of the post-spawning southward migration, and the returning northward migration, is based on data from long-term temporal patterns in catches of Spanish Mackerel by fishers and tagging studies. The following figure (Figure 11) shows commercial fishery catch data for Queensland Fish Board (QFB) landing sites averaged over 10 years. The months of peak landings begin off Cairns and Townsville in October-November (when fishers target the spawning aggregations) with peak landings moving progressively further to the south indicating the southward migration:

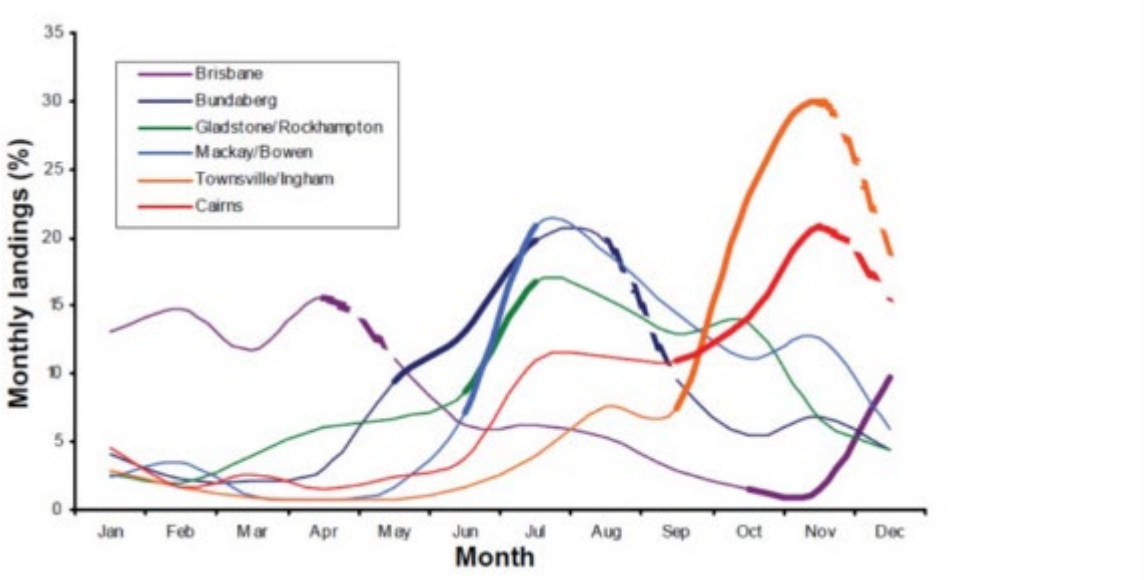


Figure 11. Monthly percentage landings (10-year average 1971-1980) from Queensland Fish Board regions. Periods of peak catch are highlighted – increasing as solid lines, decreasing as broken lines (McPherson, 2007)

Tagging studies have shown that:

- The longest migration recorded is 1000 nautical miles from northern QLD to NSW (Welch et al., 2014; Holmes et al., 2021);
- The distance Spanish Mackerel migrate southwards is positively correlated with their length, and the biggest fish are usually females (Holmes et al., 2021);

- Some fish migrate southwards at a considerable speed e.g. one fish covered 950 nautical miles in 28 days i.e. an average of 51 nautical miles/day (McPherson 1981).

The post-spawning migration is summarized in the following figure (Figure 12):

Figure 12. Representation of post spawning migration (McPherson, 2007)

The dynamics of the post-spawning migration appear to be influenced by the length/age and sex of Spanish Mackerel, water temperature, availability of prey fish, and current strength. These influences are explained in the following paragraphs.

As shown in the following table (Figure 13), the available data from tagging studies suggest that the % of fish from each age class that migrate southwards after spawning increases as the fish become older:

Geographical Distribution of Tag Returns by Year Class

NQ Releases	Returns	Year Class 0+	I+	II+
	NQ	6 (100%)	20 (90%)	13 (75%)
	SQ - NSW	0 (0%)	2 (10%)	4 (25%)
	TOTAL	6	22	17

Figure 13. Table from McPherson et al. (1981): evidence of the correlation between the increase of % of fish that migrate southwards after spawning and the age of the fish.

Data from the tagging studies found a correlation (R=0.72) between fish size and distance migrated southwards to southern Queensland and northern NSW. Larger fish are usually females (McPherson 1992), so it is likely that females (being larger at older ages) may move longer distances (McPherson 2007).

Migration southward appears to be influenced by the position and progression of the 24 °C seawater isotherm, with the southward limit determined by the position of the isotherm (Figure 14). This is based on links between the temporal changes in the position of the isotherm and temporal changes in landings of Spanish Mackerel, as suggested by the following figure:

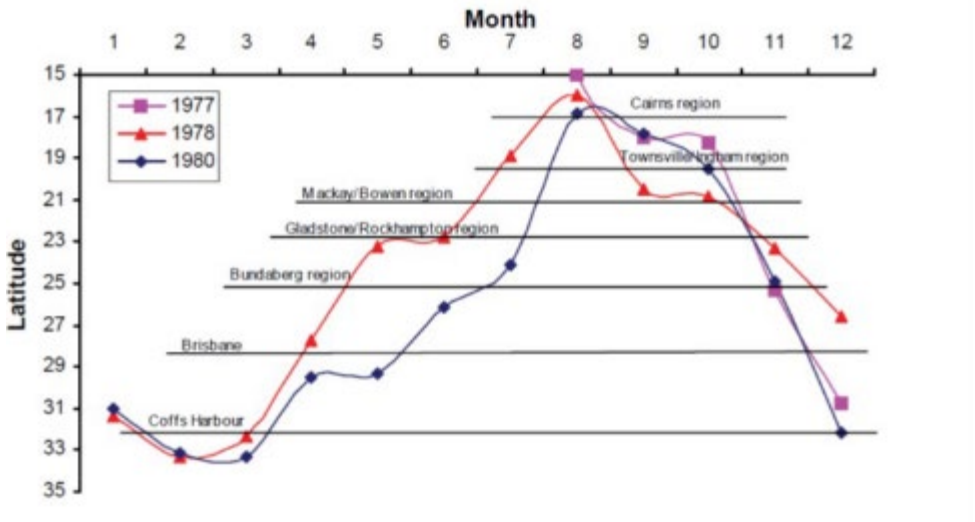


Figure 14. Position of the 24 °C isotherm at the east Australian coastline recorded by NOAA GOSCOMP system for 1977, 1978, and 1980 (McPherson, 2007).

When examined in conjunction with the data on commercial landings of Spanish Mackerel, the figure above shows:

- "Landings increased off Brisbane in December with the onset of summer and the southward movement of the 24°C isotherm.

- "It's likely that Spanish Mackerel occurred to at least Coffs Harbour (32°S), and in some years just north of Newcastle, where the isotherm may extend in some years. Fish availability off Coffs Harbour usually decreased by April.
- "Apparent fish abundance increased off Bundaberg during May-July when the 24°C isotherm was to the south of the region as did the landings in the central Queensland coast off Gladstone/Rockhampton and Mackay/Bowen. Landings at all three regions decreased when the 24°C moved through the areas and fish abundance increased to the north around Townsville/Innisfail and Cairns" (McPherson 2007 p 33).

Migratory fish return northwards during the so-called "pre-spawning season period" between May and September. This has been inferred from the historical accounts of fishers following apparently northward moving schools, and from the resultant progression of QFB landings. These fish appear to leave their summer habitat at the start of autumn. The other factor influencing the timing, and speed of the southward migration is the strength and direction of prevailing currents. Fish migrating southwards and returning northwards use the so-called 'steamer track' i.e. the Great Barrier Reef lagoon (between the coast and the GBR), rather than open ocean, offshore waters. There are south- flowing currents in the steamer track during spring-summer, at the times when fish are migrating southwards after spawning. This current moves at 21-24 nautical miles per day at this time, which would assist southward migrating fish by reducing the energy costs of migrating (McPherson 2007). Tag returns from the NSW recreational fishing game fish tagging program support the model of Spanish Mackerel returning northwards. The following figure (Figure 15, from NSW DPI 2021) shows the site of tagging (Nambucca Heads, NSW in April 2019) and re-capture (No. 10 Ribbon Reef in October 2020) of a Spanish Mackerel, representing a straight-line migration of 1037 nautical miles. The line is indicative and does not represent the actual route.

Figure 15. Albacore, Yellowfin tuna and Spanish Mackerel movements (NSW DPI 2021.)

9.5 Fishing for Spanish Mackerel

Spanish Mackerel in the east coast stock are caught by commercial (including commercial charter boats) and recreational fishers. Commercial fishers catch Spanish Mackerel by trolling with hook and line (using baited hooks or lures). Recreational fishers also use trolling with baited hooks and/or lures, and also use spearfishing (SAFS 2020).

9.5.1 Commercial Fishing

The following figure (Figure 16) shows the distribution of the commercial catch of Spanish Mackerel around Australia:

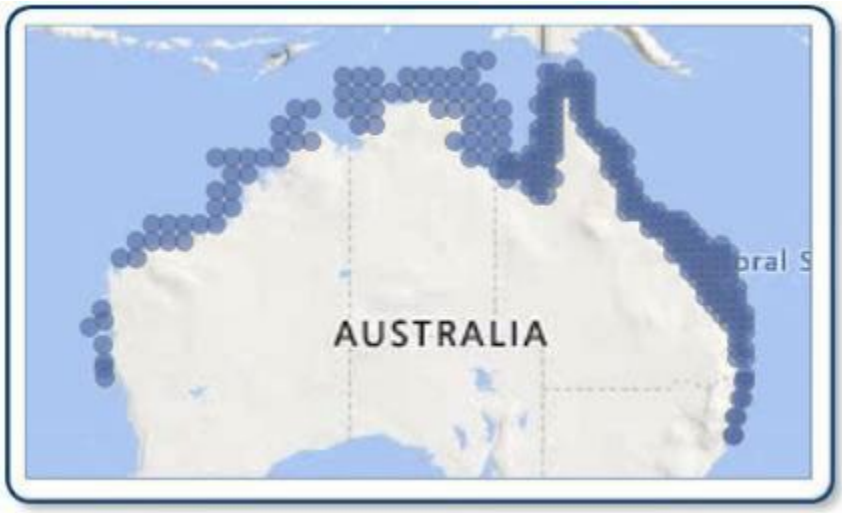


Figure 16. Geographic distribution of the commercial Spanish Mackerel catch across Australia (SAFS 2020).

In the east coast stock the majority of the commercial line catch (96%) occurs in QLD waters, with about 50% of the QLD catch occurring on reefs north of Townsville (off Lucinda) in September-November associated with the annual spawning aggregation (Figure 17). The NSW commercial catch of Spanish Mackerel occurs predominantly in late summer–autumn in northern NSW waters (Stewart et al., 2015; SAFS 2020; Tanimoto et al., 2020).

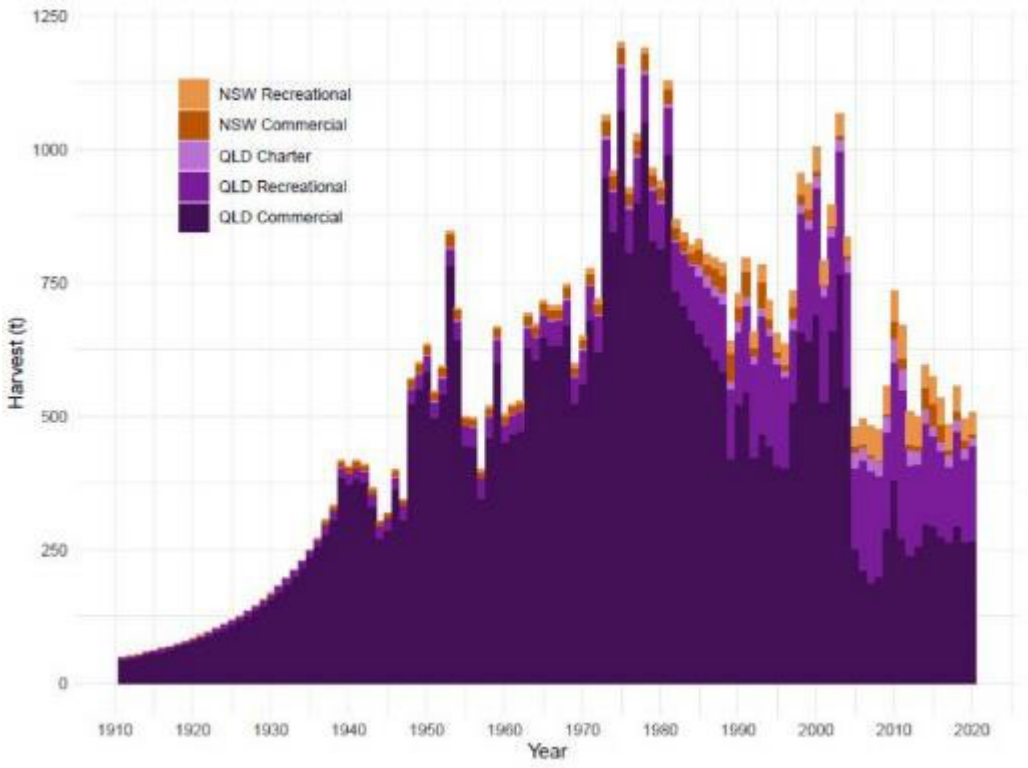


Figure 17. Annual estimated harvest from commercial, recreational and charter sectors between 1911 and 2020 for Spanish Mackerel (Tanimoto et al., 2020).

9.5.2 Environmental Influences on the Spanish Mackerel Commercial Fishery

Welch et al. (2014) tested the relationships between 4 environmental variables (SST, southern oscillation index (SOI), chlorophyll-a, river flow) and 2 aspects of the Spanish Mackerel commercial fishery on the QLD east coast (year class strength, catch-per-unit-effort CPUE). For the purposes of understanding relationships between these environmental variables and the total stock of Spanish Mackerel on the east coast, it's assumed that variations in the fishery-related variables are indicative of variations in the total population, and that these variations will also be reflected in variations in the numbers of Spanish Mackerel migrating to NSW waters (Figure 18).

The authors found:

- a negative and one-year lagged correlation between spring SST and Spanish Mackerel year-class strength (i.e. the number of new fish that each year reach the size when they are legally able to be captured in the commercial fishery).
- a positive correlation between lagged SOI and CPUE. One-year lagged SOI explained approximately 26% of variation in the annual CPUE of Spanish Mackerel over the 24- year period. La Nina events (higher values of SOI) resulted in higher catch rates; El Nino events resulted in lower catch rates. The likely explanation for this is that higher values of SOI lead to increased coastal productivity, which indirectly benefit Spanish Mackerel.
- Lagged river flow had a weak but statistically significant correlation with CPUE.
- Chlorophyll-a was not correlated with either year class strength or CPUE.

Variable	Offset	+/-	F	d.f.	P	r^2
SST	0	+	1.22	1,20	0.28	0.06
	-1	+	1.15	1,21	0.30	0.05
	+1	+	0.21	1,19	0.65	0.01
SOI	0	+	0.24	1,22	0.63	0.01
	-1	+	**7.74**	**1,22**	**0.01**	**0.26**
	+1	+	**4.64**	**1,21**	**0.04**	**0.18**
Chl-a	0	+	0.20	1,8	0.67	0.02
	-1	+	0.00	1,7	0.96	0.00
	+1	+	0.18	1,8	0.68	0.02
River flow	0	+	0.97	1,22	0.34	0.04
	-1	+	**4.81**	**1,22**	**0.04**	**0.18**
	+1	+	3.29	1,21	0.08	0.14

Figure 18. Results of linear regression of single environmental variables against CPUE in the Townsville region. Models that were statistically significant are denoted in bold (Welch et al., 2014)

9.5.3 Projected Changes in the Spanish Mackerel Fishery from Climate Change

Welch et al (2014) made the following general predictions for Spanish Mackerel (Figure 19):

Species	Key potential effects of climate change (based on 2030 projections)
Spanish mackerel	• Increasing strength of the EAC likely to cause a poleward range extension (+ve for SE Qld/NSW & SW WA) • Increasing SST could also cause a poleward shift of the main spawning (and fishery) area on the east coast and/or lower east coast population sizes (+/-)

Figure 19. Table from Welch et al. (2014). Potential effects of climate change on different species of northern Australian commercial fishes.

In a more recent study, Champion et al. (2022) (Figure 20) made the following projections about the Spanish Mackerel fishery:

- The authors used the proportion of the year that target species are available to fishers as a proxy for fishing opportunity, and this proportion is based on the temporal persistence of suitable oceanographic habitat in a region.
- The persistence of suitable habitat conditions for Spanish Mackerel is projected to increase by 1.5 months between 2020 and 2050 in the Hawkesbury Shelf bioregion, 1 month in the Batemans Shelf bioregion, and 1 month in the Twofold Shelf bioregion.
- The projected increase in persistence was significantly associated with projected changes in sea

surface temperature, chlorophyll a concentration, and sea surface height with model performance improved when seascape topographic variability was accounted for.

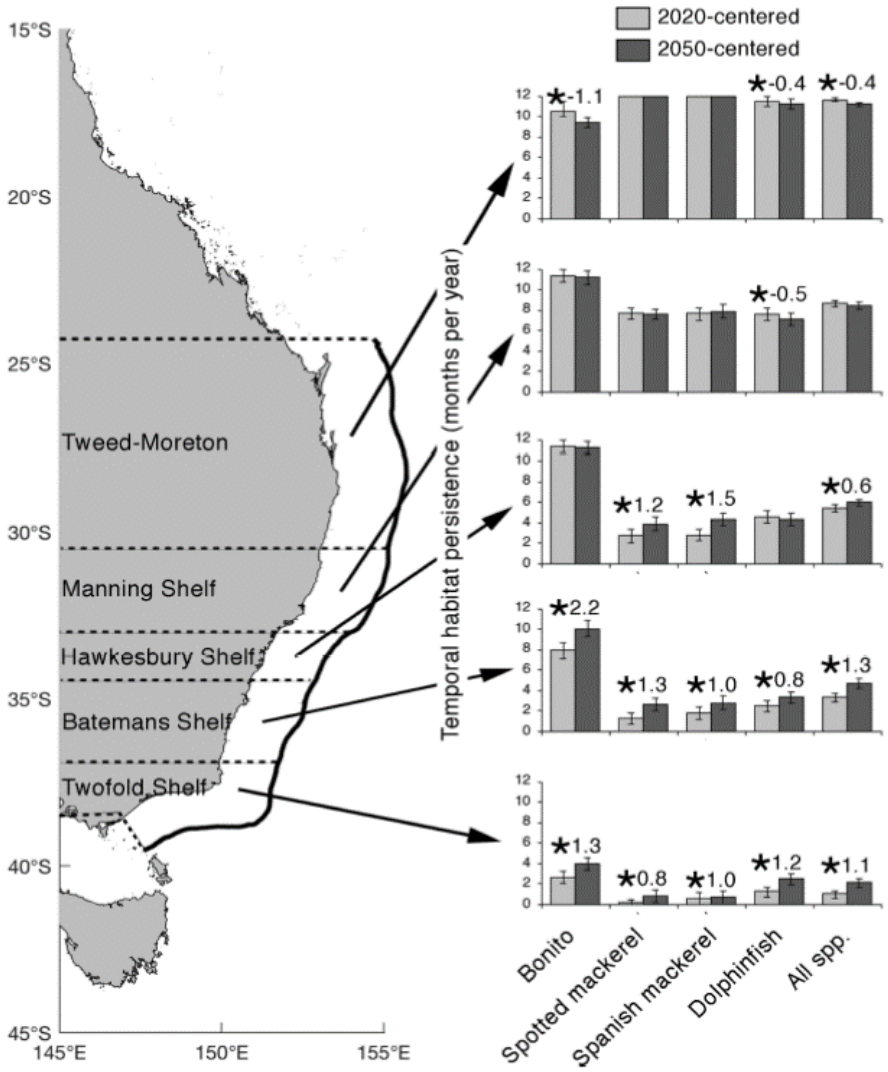


Figure 20. Comparison of the temporal persistence (months per year) of suitable environmental habitat between 10-year averages centred on 2020 and 2050 for each study species and the average for all species (All spp.) within eastern Australian bioregions. Significant differences (at alpha level 0.05) in temporal habitat persistence between 2020- and 2050-centered average are denoted by asterisks, with the size of the mean difference indicated by the adjacent values. Dark line represents the offshore bioregional boundary. (Champion et al., 2022)

9.5.4 Recreational Fishing for Spanish Mackerel in NSW

In NSW, the recreational fishery for Spanish Mackerel is substantially larger than the commercial fishery (see table in Figure 21 below):

Sector	2001	2014	2020
QLD Commercial	525 945 (66.4%)	299 872 (50.3%)	266 565 (52.5%)
QLD Charter	20 207 (2.6%)	30 041 (5.0%)	16 650 (3.3%)
NSW Commercial	3 384 (0.4%)	39 703 (6.7%)	7495 (1.5%)
NSW Recreational	45 535 (5.7%)	42 522 (7.1%)	40 626 (8.0%)
QLD Recreational	189 577 (23.9%)	164 229 (27.5%)	166 272 (32.8%)
QLD discard mortality	7748 (1.0%)	20 037 (3.4%)	9778 (1.9%)

Figure 21. Harvest shares per sector (including "QLD discard mortality") expressed in kilograms with annual percentages (Tanimoto et al., 2020).

The recreational catch of Spanish Mackerel in NSW varies by year, and by region in the following ways (Tanimoto et al 2020):

- Spanish Mackerel are a very minor component of the total recreational catch of finfish in NSW: in 2017-18 the total catch of Spanish Mackerel (3301 fish) represented 0.05% of the total catch of finfish in NSW.
- 2017-18: amongst 90 different types of saltwater finfish caught in NSW, Spanish Mackerel ranked 49/90.
- 2017-18: 85% of Spanish Mackerel caught were kept; 87% were caught from a boat; 6% were caught by divers (i.e. spearfishers); 88% were caught in the northern region (QLD border-Stockton Beach (just north of Newcastle)); 12% were caught in the central region (Stockton Beach- Shellharbour (just south of Wollongong)); 0 were caught in the southern region (Shellharbour to Victorian Border).
- 2017-18 compared to 2013-14: the total catch of Spanish Mackerel in 2017-18 was 48% of the total catch in 2013-14; the total number of Spanish Mackerel kept in 2017- 18 was 44% if the total number kept in 2013-14.
- 2019-20: only 171 Spanish Mackerel were caught in 2019-20, which was 3% of the total catch for 2013-14 and 6% of the total catch for 2017-18.

10 Comparison of CTX rapid test kits

10.1 Background

The review of ciguatoxin (CTX) analytical techniques conducted at the start of this project identified a commercially available ELISA kit as a promising technique for the screening of fish extracts for the presence of ciguatoxins. Specific to the detection of Pacific ciguatoxin type 1 (P-CTX-1), the 96-well microplate format allows for multiple samples to be analysed simultaneously and P-CTX-1 detected to very low concentrations (advertised limit of detection = 0.02 or 0.0005 ng P-CTX-1/mL for absorbance and fluorescence techniques, respectively). A brief initial trial with this test kit yielded promising results and the following chapter describes the performance of the ELISA test kit and suitability for detection of CTX in Spanish Mackerel in more detail, including:

- An assessment of key ELISA performance criteria to refine the assay protocol. This includes comparison of potential interference from different sample solvents and diluents, as well as a preliminary determination of the P-CTX-1 limit of detection (LOD), and quantification of the variability within and between assay runs.
- Comparison of P-CTX-1 detection with the ELISA test kit to that of LC-MS and the N2a neuroblastoma cell line assay using a known subset of CTX positive samples.
- Consideration of the ELISA logistics, including time restraints and estimated time of analysis, as well as further opportunities to fine-tune these.

10.2 Methods

10.2.1 Standard ELISA protocol

ELISA test kit components

P-CTX-1B and P-CTX-51-OH-3C ELISA test kits were obtained from Bold Biotechnology, Japan and shipped to Australia via courier. Test kits were kept refrigerated during transport to keep storage temperatures within the manufacturer's recommendations (as verified with temperature loggers during transport). Upon arrival, all test kits and components were stored at 4°C until analysis. Each test kit includes 2 x 96-well microplates (pre-coated with capture antibodies), plate wash buffer solution, sample diluent, detection anti-body (anti-CTX1B-ALP), antibody diluent and 100 µL of Japanese P-CTX-1B standard at 5 ng/mL.

ELISA protocol

The ELISA protocol closely followed that outlined in the manual supplied with the test kits [1]. Firstly, the P-CTX-standard was diluted in assay diluent to give 0.00156 – 0.1 ng/mL P-CTX-1B to generate a standard curve to which samples with unknown P-CTX concentrations could be compared (see statistical analysis below for details). A volume of 100 µL of either P-CTX-1B standard, blank (assay diluent only) or sample (fish extract) was added to triplicate microplate wells. The microplate was covered

with an adhesive plastic sheet and subsequently incubated in the dark at room temperature (25 °C) for 30 minutes. The contents of the microplate were then discarded and the plate washed three times with the supplied wash buffer (200 µL per well). After the final washing step, 100 µL of the anti-CTX1B-ALP solution were added to each well and the plate incubated for a further 30 minutes at room temperature. After this time, the plate was washed three times with the supplied wash buffer and 100 µL of AttoPhos®AP Fluorescent Substrate System (S1000, Promega) added to each well. Following another 30 min of incubation at room temperature, fluorescence was quantified using a microplate reader (BMG Labtech Fluostar Optima) at excitation and emission wavelengths of 430-10 and 550-10 nm, respectively.

10.2.2 Method Optimization

Linearity of dilution

Sample diluents

To determine if different sample diluents influenced the ELISA fluorescent signal, the 5 ng/mL P-CTX- 1 in dimethyl sulfoxide(DMSO) standard supplied with the Japanese ELISA test kit, was diluted to 0.01, 0.050 and 0.080 ng/mL P-CTX-1 with either 5% methanol (MeOH), 80% MeOH and compared to a standard curve prepared with the Japanese P-CTX-1 standard diluted to 0-0.100 ng/mL using the assay diluent supplied with the test kit. These dilutions were then analysed following the P-CTX-1 ELISA protocol described above.

Sample solvents

Both DMSO and 80% MeOH are commonly used to resuspend fish extracts in the final step of ciguatoxin extraction procedures. The extraction & LC-MS method employed in this project requires resuspension of the sample in 80% MeOH as the final step in preparation for LC-MS analysis. To facilitate the comparison of LC-MS and ELISA results, it was deemed preferable to also use 80% MeOH as the solvent for the ELISA. To determine if there was any impact on the final P-CTX-1 value obtained with the ELISA by using either solvent, equal aliquots of 5 Spanish Mackerel samples spiked with P- CTX-1 were evaporated to dryness under nitrogen gas at 55 °C and resuspended in either 80% MeOH or DMSO, diluted x20 in assay diluent and tested on the P-CTX-1 ELISA.

ELISA performance parameters

Limit of detection

To determine the limit of detection (LOD), data were confirmed to be normally distributed (Shapiro Wilk's test) and individual pairwise Welch's t-test conducted for 11 different assay runs to determine if the lowest P-CTX-1 standard (0.94×10^{-3} ng/mL) could be reliably distinguished from the blank.

Accuracy & precision

To estimate the accuracy of the P-CTX-1 ELISA test, the percentage recovery of French Polynesian P- CTX-1 standards was calculated over six individual ELISA runs conducted over multiple days. This was achieved by fitting a 5 parametric logistic equation to the P-CTX-1 standards for each ELISA run and comparing the such estimated concentrations of standards to the expected concentration as follows:

Percentage recovery = [observed]/[expected]*100

Inter-assay coefficients of variability were calculated across 11 individual assay runs for the highest (60 x 10^{-3} ng/mL P-CTX-1) and lowest (0.94 x 10^{-3} ng/mL P-CTX-1) concentrations of the French Polynesian P- CTX-1 standards.

Variability between assay runs

To assess the variability between repeat ELISA assay runs, 12 P-CTX-1 positive samples that had previously been confirmed to contain CTX with LC-MS were tested in three different assay runs conducted on three different days. As per the revised sample preparation protocol, the samples were resuspended in 80% methanol and diluted in assay diluent by a factor of 20 prior to testing on the ELISA.

A second set of trial runs was conducted to determine if the assay protocol was subject to plate drift (measure of sample variability within the same plate). Ten identical P-CTX-1 positive sample dilutions were prepared in assay diluent as above and pipetted into the multi-well plate at the start and end of the pipetting step. These ten samples were tested across multiple assay runs, with 3 samples per plate.

10.2.3 Method comparison

To compare P-CTX-1 concentrations estimated via the ELISA to other analytical methods, 10 Spanish Mackerel liver and 10 flesh extracts were tested with the ELISA, N2a neuroblastoma cell line assay and LC-MS. Ciguatoxin extractions were performed as described by Murray et al. (2018)), with the following modifications. Extracts were prepared from 10 g of fish flesh (rather than 5 g in original method) and final extracts resuspended in 400 instead of 200 µL of 80% methanol. The solvent volumes for all other SPE conditioning and eluting steps remained the same. This scale-up was necessary to obtain sufficient extract to test across all three techniques. The final 400 µL of extract were divided into two lots of 100 µL (ELISA and N2a analysis) and one aliquot of 200 µL for LC-MS/MS analysis. These were taken to dryness at 55˚C under a stream of nitrogen and shipped at ambient temperature before being resuspended in either 80% methanol (LC-MS/MS and ELISA) or N2a cell line growth medium (Viallon et al., 2020)). LC-MS/MS analysis were performed at Cawthron Institute, New Zealand, N2a analysis conducted at the Institute Louis Malardé in French Polynesia following protocols outlined in Viallon, et al.(2020). ELISAs were run at the Institute for Marine and Antarctic Studies in Hobart, Australia.

The majority of the samples tested across these assays were confirmed to contain P-CTX-1 in a previous study via LC-MS/MS (10) and have been re-analysed here. A further eight naturally CTX contaminated positive fish flesh samples (also used as quality control samples during the sample screening) were analysed to obtain additional data points to compare both the LC-MS and ELISA detection methods. Spanish Mackerel samples collected during the 2020-2021 and 2021-22 fishing seasons were resuspended in 80% methanol and diluted by a factor of 20 in assay diluent prior to testing for P-CTX-1 with the ELISA. Four Spanish Mackerel flesh and four liver samples spiked with P-CTX-1 standard and nine positive controls prepared from CTX positive Spanish Mackerel flesh were also tested on the ELISA as QC samples.

10.2.4 Data analysis

Standard curve and ciguatoxin estimation

A five parameter logistic regression was fitted to the raw fluorescence values of the P-CTX standards for each individual assay run using the statistical package drc in R https://www.r-project.org/. This standard

curve was generated for each individual ELISA plate and employed to estimate CTX concentrations of individual samples tested in that plate. One-way analysis of variance (ANOVA) where employed to test for differences in the fluorescence signal between different solvent blanks (i.e. respective solvents only, no CTX).

10.3 Results & Discussion

10.3.1 Method Optimization

Linearity of dilution

The fluorescent signal obtained with the P-CTX-1 ELISA differed significantly between standard diluents (Figure 22). As expected, the fluorescent signal of the Japanese P-CTX-1 standard diluted in assay diluent increased with increasing P-CTX-1 concentration, allowing for a standard curve of best fit to be calculated. However, the fluorescent signal of the same standard diluted in 5% MeOH was significantly lower and indistinguishable from the blank when using 80% MeOH as diluent. Together with the fact that there were no significant differences observed between the respective solvent blanks (ANOVA, $F_{2,6}$ = 2.14, p=0.199), this suggests that higher MeOH concentrations are interfering with P-CTX-1 estimation by quenching the fluorescent signal when P-CTX-1 is present.

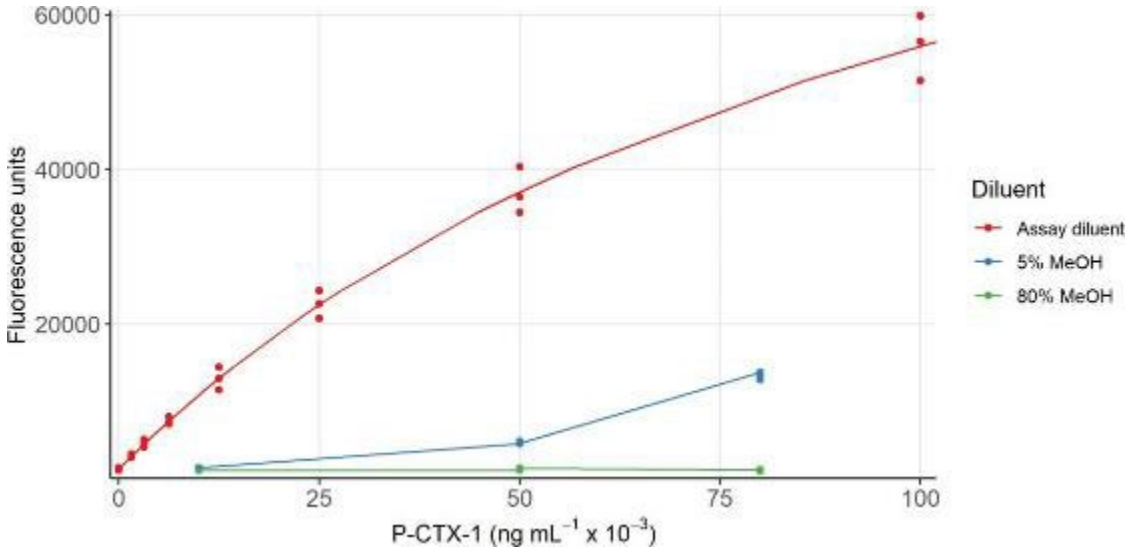


Figure 22. Fluorescent signal of Japanese P-CTX-1 standard diluted in either assay buffer (red), 5% MeOH (blue) or 80% MeOH (green).

Based on the dilution trials with the Japanese P-CTX-1 standards, fish extracts resuspended in 80% MeOH at the end of the CTX extraction process require dilution in assay diluent before testing them with the ELISA. The manufacturer's instructions supplied with the ELISA test kit recommend a 20-fold dilution for samples resuspended in DMSO, but no guidance is provided for the use of 80% MeOH as the sample solvent (as is used for LC-MS). When comparing the final estimate of P-CTX-1 across five distinct samples (as shown in Figure 23, ELISA 1-5), no notable differences were observed in comparison to the control (Figure 23, ELISA 6). The samples, which were resuspended using either DMSO or 80% MeOH as solvents and then diluted 20-fold in assay buffer (as illustrated in Figure 23), showed consistent results.. From this point onwards, all samples were dissolved in 80% MeOH and diluted 20-fold in assay diluent prior to analysis.

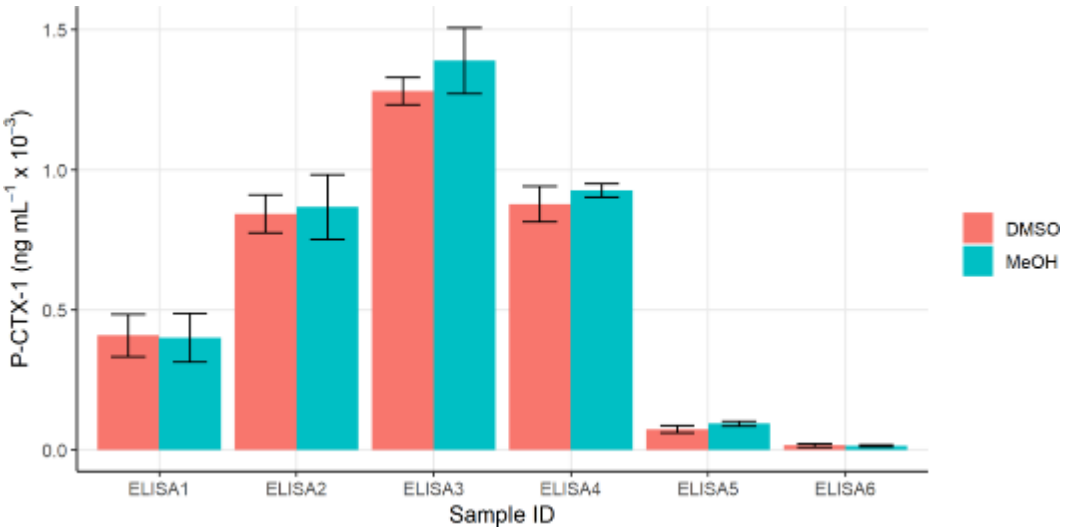


Figure 23. P-CTX-1 concentrations detected with the ELISA in Spanish Mackerel extracts dissolved in either DMSO (red bars) or 80% MeOH (green bars) and diluted 20x in assay buffer. P-CTX-1 concentrations of these 5 samples and the control (ELISA 1-6) were estimated based on P-CTX-1 standard curves generated with the corresponding solvents. Error bars represent 1 standard deviation around the mean (n=3).

10.3.2 ELISA performance parameters

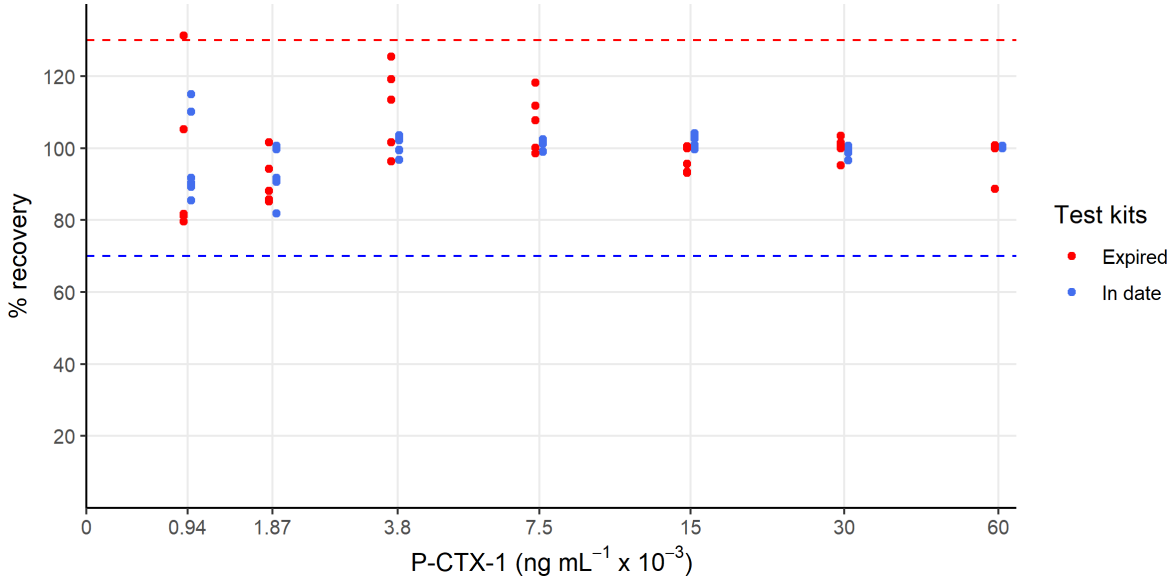
Limit of detection

The limit of detection (LOD) for the commercial ELISA test kit was experimentally determined to be between 0.5 - 0.94 x 10^{-3} ng P-CTX-1/mL (equivalent to 0.00094 ppb). To test if our assay system could replicate the same level of detection, the original experimental design included an extra P-CTX-1 standard just above the LOD advertised by the manufacturer (0.56 pg x 10^{-3} ng P-CTX-1/mL). However, later purity analysis of the French Polynesian standard used for comparison to LC-MS and N2a results indicated a different level of purity. The lowest P-CTX-1 standard concentration tested therefore was below the advertised LOD. At this lower concentration (0.47 x 10^{-3} ng P-CTX-1/mL), the blank could only be distinguished from the standard 50% of the time (4 assay plates, Welch's t-test p = >0.05). However, at the next highest standard concentration tested (0.936 x 10^{-3} ng P-CTX-1/mL), the fluorescent signal could be reliably distinguished from that of the blank (Welch's one-sided t-tests conducted on 11 individual assay runs, p = <0.028). This places the actual limit of detection between 0.47 and 0.94 x 10^{-3} ng P-CTX- 1/mL. Since no concentrations in between were tested, we employed an LOD of 0.94 as a cut off point for all data analysis going forward, noting samples that returned values between the here determined LOD and the LOD supplied by the manufacturer (0.5 x 10^{-3} ng P-CTX-1/mL). Taking into account the 20-fold dilution required to eliminate sample solvent interference, the estimated LOD translates to 0.01-0.0188 ng/mL P-CTX-1 in the original sample extract (i.e. prior to dilution), which in turn translates to 0.002- 0.0037 µg P-CTX-1 per kg of fish tissue (using the here employed extraction method of Murray et al. (2018)). While this is well below the US FDA guidance level of 0.01 µg P-CTX-1 per kg of fish tissue, testing of additional low concentration P-CTX-1 standards and diluted fish flesh extracts is recommended to more accurately define the true limit of detection. This would also provide additional confidence for establishing a limit of quantification based on the percentage recovery of standards (75 – 125% recovery are generally accepted (e.g. Leonardo et al., 2020).

Accuracy, precision & use of expired test kits

Examination of the percentage recovery of CTX standards (i.e. comparison of the observed vs. expected P-CTX-1 concentration), revealed that most observations were within 30% of the expected value (Figure 24). This value (30% of expected value) is generally considered satisfactory for ELISA assays , and it is typical for standard concentrations at the lower and higher end of the standard curve to exhibit a larger variation in percentage recovery. While the highest P-CTX-1 concentration tested here (60 x 10^{-3} ng/mL) showed excellent recovery (less than 20% variation), that of the lowest standard (0.94 x 10^{-3} ng/mL) exceeded 30%. At the next highest P-CTX-1 standard concentration, this variation was considerably reduced.

As samples became available throughout the fishing seasons, some leftover test kits were employed to test additional field samples (see Chapter 11). These test kits had surpassed their expiry date by 2 months, yet performed close to the 30% P-CTX-1 standard recovery (see Figure 24 for comparison between expired and in date test kits). When considering additional performance parameters, both in date (n=6) and expired test kits (n=5) performed almost identical. Inter-assay coefficients of variation (measure of the variation of the highest and lowest P-CTX-1 standard concentration tested between different assay runs) were 11.95 and 11.3% CV, for in date and expired test kits, respectively (<15% CV is generally acceptable for ELISAs (17, 18)). Pairwise comparison of the blank and lowest P-CTX-1 standard concentration tested confirmed the above experimentally determined LOD for both in date and expired test kits. It is noteworthy that the in-date test kits generally presented with a lower variation in the percentage recovery than the expired test kits (<±20% for in date test kits). This high level of accuracy is commonly only required for quantitative



ELISAs employed in medical diagnostics .

Figure 24. Percentage recovery of P-CTX-1 standards (recovery = [observed]/[expected]*100) across the standard range of P-CTX-1 concentrations (0.94-60 x 10^{-3} ng/mL) assayed across 11 ELISA runs (mean of triplicate standards). The lower and upper levels of generally acceptable % recovery are indicated by the dashed blue and red lines, respectively. Blue and red points indicate the % recovery of individual assay runs of in date and expired test kits, respectively.

Variability between assay runs

At lower P-CTX-1 concentrations (<0.1 ng/mL), the ELISA yielded consistent results, whereas variability increased at higher P-CTX-1 concentrations (up to 0.28 ng/mL difference between maximum and minimum P-CTX-1 estimates in extract from fish sample number "*AIMS12L"*). This reflects variability between ELISA runs, as well as potential pipetting errors during

sample preparation (samples need to be diluted fresh before each run). Across the three replicate assay runs, the ELISA reliably detected the presence of P-CTX-1 in all of the 12 P-CTX-1 positive samples. For 2 samples with lower P-CTX-1 concentrations (*AIMS10F* and *AIMS6L*), two assay runs yielded quantifiable results (all triplicate wells in each assay >LOD), while the results of the third run were not as conclusive (i.e. one or two wells below the LOD, Figure 25).

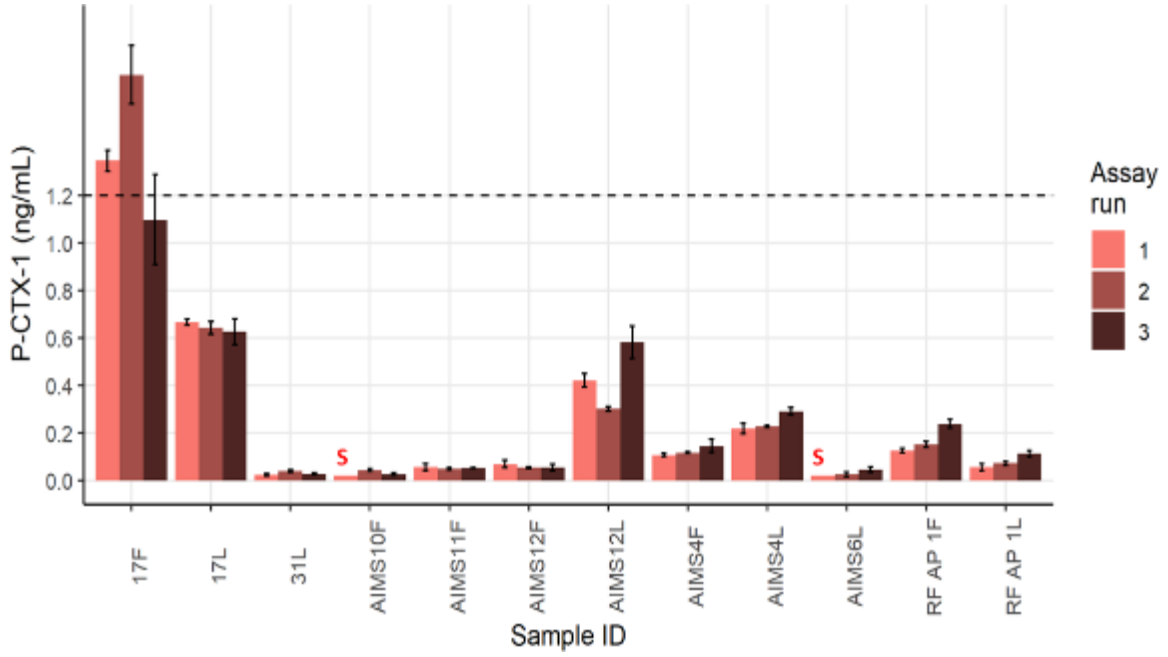


Figure 25. Variation in P-CTX-1 concentration (ng/mL) of samples diluted and tested on 3 different days (each coloured bar represents a single assay run conducted on a separate day). The dashed line represents the upper limit of quantification (1.2 ng/mL equivalent to the highest P-CTX-1 standard tested). Error bars represent 1 standard deviation around the mean and S indicates suspect samples (where one or two of the triplicate wells in the assay run returned a result below the LOD).

A likely contributing factor to the variation observed between assay runs was plate drift. To pipette all standard solutions and CTX standards into individual wells takes approximately 10-20 minutes. Samples added to the microplate at the start of pipetting would therefore have considerably more time to bind to the P-CTX-1 capture antibodies in the microplate wells than those pipetted later in the same run. Testing 10 samples that were pipetted at the start and end of the identical plate (3 samples tested per plate), we found that this was indeed the case, particularly at P-CTX-1 concentrations exceeding 0.1 ng/mL (up to 0.5 ng/mL higher when comparing those added at the start and end of the pipetting run). This effect was not as pronounced for samples with lower (<1 ng/mL) levels of P-CTX-1 (Figure 26). If the assay is to be used quantitatively, care should therefore be taken to reduce the time of pipetting (e.g. pipette all samples into a spare microplate first and transfer multiples at once across to the assay plate with a multichannel pipettor). For qualitative P-CTX-1 detection (presence/absence), this plate drift appears to have less of an effect (noting the limited number of low P-CTX-1 samples tested here).

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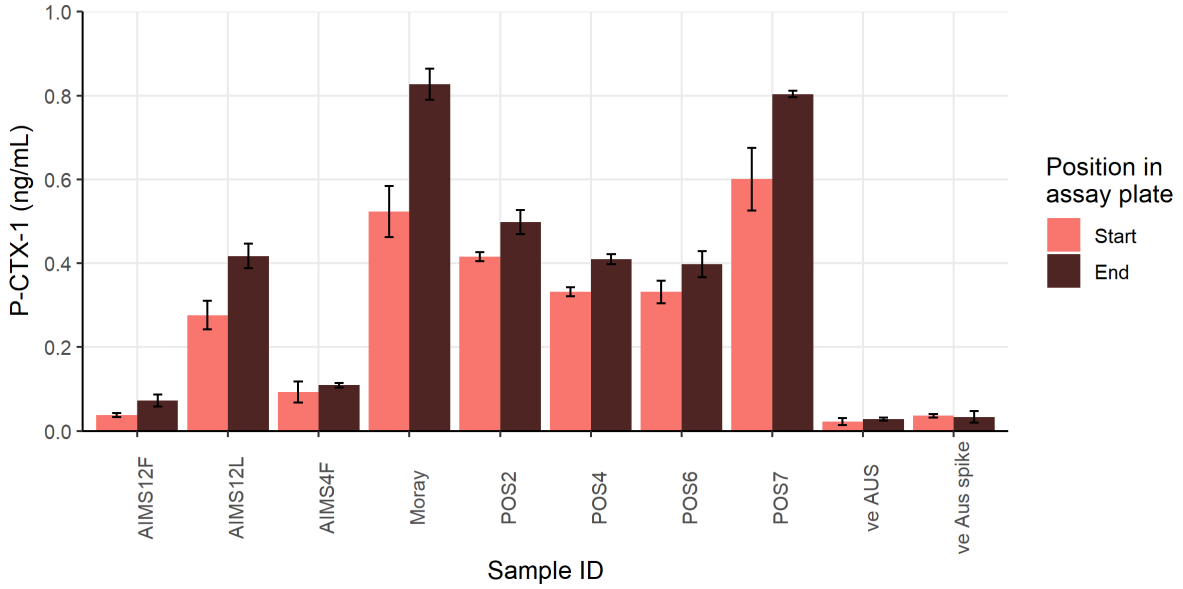


Figure 26. Plate drift across multiple CTX positive samples that were pipetted into the ELISA microplates at the start (red bars) or end (brown bars) of the same microplate. Error bars represent 1 standard deviation around the mean.

10.3.3 Method comparison

Analysis of 18 different Spanish Mackerel liver (n=9) and flesh extracts (n=9) indicated a good general agreement in CTX detection between the ELISA, N2a and LC-MS analytical techniques (Table 2). The ELISA method detected P-CTX-1 in all flesh and liver samples where either N2a or LC-MS reported quantifiable concentrations. Generally, P-CTX-1 concentrations estimated with the ELISA were in good agreement to those quantified by either LC-MS or N2a (r^2 of 0.68 and 0.98, respectively, Figure 27). This close correlation is particularly noteworthy when considering that the individual analysis where conducted in 3 different countries by three different operators that resuspended the samples in different analytical solvents/media. P-CTX-1 concentrations estimated for the positive control samples via LC-MS and ELISA (Table 2) also exhibited a good correlation, particularly when taking into account the above-described plate drift for the ELISA assay (r^2 = 69, data not shown). The variation in the estimated P-CTX-1 concentrations among these eight different positive control samples was highly comparable for both the ELISA and LC-MS methods (44 and 48% CV, respectively).

However, in the two samples where both the ELISA and N2a assay returned the highest P-CTX-1 concentrations (AIMS12L and AIMS4L), LC-MS did not detect the presence of P-CTX-1. Two possible explanations for this observation include either the presence of sample impurities that may have interfered with LC-MS/MS detection, or the presence of a compound with cytotoxic properties that structurally sufficiently resembles P- CTX-1 to bind to ELISA detection antibodies. Throughout this pilot work, the N2a assay consistently reported higher estimates of P-CTX-1 than either LC-MS or ELISA (Table 2). This trend has been observed throughout other comparison studies (e.g. Leonardo et al., 2020) and can be attributed to the specificity of the N2a assay. As a cell line bioassay, the N2a is not specific to P-CTX-1 and other sodium channel blocking substances, such as other CTX analogues or other cytotoxins (e.g. maitotoxins) may contribute to the observed P-CTX-1 like activity in a sample. At the same time, the ELISA antibodies are reportedly highly specific for P-CTX-1, with no cross reactivity to the structurally related P-CTX-3C or 51-hydroxyCTX-3C, nor brevetoxin, okadaic acid or maitotoxin (Tsumuraya et al., 2019). Given the high concentration indicated by both ELISA and N2a, it appears most likely that this is a true P-CTX-1 detection.

Another interesting case is presented by the liver and flesh of a single fish ("*FRDC 17",* Table A6), where neither LC-MS nor N2a had indicated the presence of CTX, while the ELISA returned the highest P-CTX-1 concentrations reported for any samples (0.13 and 0.28 µg P-CTX-1/kg for liver and flesh, respectively). Given the high concentration estimated by the ELISA, it appears unlikely that this is a true detection and may be considered as a false positive on the ELISA, as these higher concentrations should be well within the LC-MS and N2a level of detection. This high fluorescent signal could be due to fluorescent compounds intrinsic to the sample/fish or the presence of a compound closely related to P-CTX-1 that was bound by the detection antibodies. Since the detection antibodies are highly specific to each wing of the P- CTX-1 molecule, fluorescence interference appears most likely. Coincidentally, we also observed high background fluorescence in a ciguatoxin extract prepared from a fish curry that had previously been confirmed via LC-MS to contain P-CTX-1. Even when diluted 50-fold, this extract completely quenched the ELISA fluorescence signal, likely due to the high turmeric content of the sample. Overall, the high fluorescent signals for extracts prepared from fish number "*FRDC 17*" appear to be an isolated observation. No such high P-CTX-1 fluorescence signals were obtained when screening >120 tissue extracts from the 2021-22 season (see Table A6).

While it is possible that low level background fluorescence could contribute to over estimation of low level P-CTX-1 detections in certain samples, the ELISA and the N2a results were in excellent agreement for samples containing low levels of P-CTX-1 (Table 2, Figure 27). Both these assays identified the presence of low, but quantifiable concentrations of P-CTX-1 in 3 additional samples (<0.022 µg/kg), where no P-CTX was detected via LC-MS. Furthermore, the N2a assay identified five additional samples where trace quantities of CTX around the limit of detection may be present (designated as "suspect" samples). The ELISA returned quantifiable results for 4 of these samples, quantified P-CTX-1 in another sample where none was detected by N2a and identified a further 3 samples as suspect, where the N2a had not detected any CTX- like activity. Rather than being an artefact of sample background fluorescence, lower level detections with the ELISA might just be due to the comparatively lower level of detection for this assay (i.e. compare LOD of 0.002-0.0037 µg P-CTX-1 per kg of fish tissue for ELISA and 0.0096-0.17 µg P-CTX-1/kg for the N2a)).

Table 2. Comparative detection of P-CTX-1B in fish flesh and liver extracts prepared from Spanish Mackerel and analysed with the ELISA and N2a assays, as well as LC-MS. Both ELISA and N2a results are the mean of triplicate analysis conducted on three separate days. Numbers represent the mean P-CTX-1 concentration ± 1 standard deviation. NA represents samples not analysed on the N2a assay and suspect represents samples where some ciguatoxin like activity above the respective level of detection was detected in only one or two replicate wells of the ELISA and N2a assay runs. * denotes ELISA results for fish extracts where only enough sample volume for a single assay run was available (i.e. not analysed in triplicate runs). Note that samples collected during the 2015 fishing season (denoted here with **) (10) were reanalysed via LC-MS at the time of N2a & ELISA analysis to account for any possible sample degradation since the initial CTX detection in 2015.

Tissue	Sample ID	Sample details	ELISA (ng/mL)	N2a (ng/g)	LC-MS (ng/mL)
Flesh	AIMS4F	2015 season**	0.12±0.02	0.06±0.00	0.063
Flesh	AIMS12F	2015 season	0.06±0.01	0.04±0.03	0.057
Flesh	AIMS11F	2015 season	0.05±0.00	0.03±0.02	0.031
Flesh	AIMS10F	2015 season	0.03±0.01	0.02±0.01	0.023
Liver	AIMS12L	2015 season	0.43±0.14	0.22±0.01	Not detected
Liver	AIMS4L	2015 season	0.25±0.04	0.14±0.01	Not detected
Flesh	RF AP 1F	2015 season	0.17±0.06	Suspect	Not detected
Liver	RF AP 1L	2015 season	0.08±0.03	0.07±0.003	Not detected
Liver	AIMS6L	2015 season	0.03±0.01	Suspect	Not detected
Liver	CFC 11 L	2015 season	0.03±0.01*	Suspect	Not detected
Liver	FRDC 31L	2021-22 season	0.03±0.01	Not detected	Not detected
Liver	FRDC 123L	2021-22 season	Suspect	Not detected	Not detected
Flesh	FRDC 132F	2021-22 season	Suspect	Not detected	Not detected
Flesh	FRDC 133F	2021-22 season	Suspect	Not detected	Not detected
Liver	AIMS11L	2015 season	Not detected	Suspect	Not detected
Flesh	AIMS6F	2015 season	Not detected	Suspect	Not detected
Flesh	CFC 11 F	2015 season	Not detected*	Not detected	Not detected
Liver	FRDC 8L	2021-22 season	Not detected	Not detected	Not detected
Flesh	POS 1	Positive control	0.6±0.07	NA	0.279
Flesh	POS 2	Positive control	0.47±0.01	NA	0.238
Flesh	POS 3	Positive control	0.46±0.03	NA	0.191
Flesh	POS 4	Positive control	0.23±0.02	NA	0.236
Flesh	POS 5	Positive control	0.25±0.03	NA	0.161
Flesh	POS 6	Positive control	0.31±0.03	NA	0.269
Flesh	POS 7	Positive control	0.68±0.03	NA	0.496
Flesh	POS 8	Positive control	0.79±0.03	NA	0.532

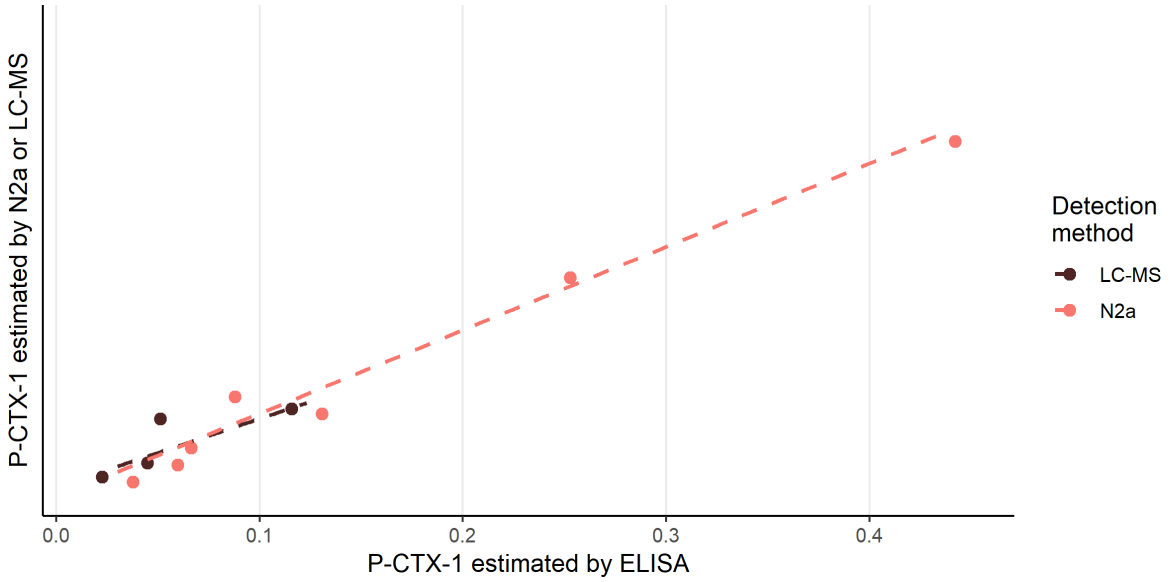


Figure 27. Correlation between P-CTX-1 estimated with the N2a assay or LC-MS (y-axis) and those estimated with the ELISA (x-axis).

10.3.4 Indicative cost and logistics of CTX analysis

Indicative cost & materials

The ELISA test kit costs AUD $1,250 per kit (2021 $), and contains 2 antibody coated assay plates. The number of samples that can be run in each plate is dependent upon the number of P-CTX-1 standard concentrations to be included in each assay run. If detection, as well as quantification of P-CTX-1 is the desired output, the manufacturer recommends using 8 different standard concentrations (as was done here for this pilot work). Using the assay for screening purposes (i.e. presence/absence of P-CTX-1) may require fewer standard concentrations to be run, but at an absolute minimum should include a blank, one low and one high P-CTX-1 standard for quality control purposes. Based on our experiences with the assay, analysis of each sample across triplicate wells proved sufficient to obtain coefficients of variation within the manufacturer's guidelines (<15% CV). No less than three replicate wells should be used for each sample. In these configurations, each assay plate could run 24-29 samples (i.e. 48-58 samples per test kit). Taking into account the materials required for each assay run (general consumables & Attophos substrate for fluorescent detection technique, as well as labour for analysis & refrigerated international shipping of test kits), this translates into an estimated cost of $40-50 per sample (see Table 3, this does not include labour for extraction, discussed under *Timing* below). Other key logistical requirements include access to clean bench space (1 x 4m), a fluorescence detection capable plate reader (~$40,000-50,000 if purchased new) and general laboratory equipment, including an accurate multichannel pipette (~$3000-5,000) and refrigeration for reagent/sample storage (1x1 m fridge space). A skilled technician is required to run the ELISAs and report on results.

Timing

While an individual ELISA assay can be conducted by a skilled operator in approximately 4 hours, sample preparation requires a substantial time investment. This is due to the lengthy extraction process required to purify the fish extract for analysis. For this pilot work, we employed the revised extraction protocol developed by Murray et al., (2018) to directly compare results across multiple analytical techniques, including LC-MS. An attractive proposition is the use of simplified extraction protocols that do not necessitate as many lengthy samples clean up steps as those required for LC-MS analysis (e.g. the here employed LC-MS protocol requires approximately 6.5 hours for 10 samples). While the limit of detection for these cruder tissue extracts remains unknown for the ELISA, an earlier, less sensitive ELISA was able to be conducted on crude extracts without

apparent protein or lipid interference (Tsumuraya et al., 2018) . This would present significant cost-savings and could allow for potential use of the ELISA as a sample pre-screening tool, where positive detections could be followed up with either LC-MS or N2a analysis. Considering the cost of ELISA analysis alone (~$40-50 per sample for analysis only), such an approach will not be feasible for blanket screening of fish before market. Instead, a more feasible approach for using these assays in a research/environmental monitoring capacity (e.g. identification & monitoring of sentinel reef fish in ciguatera hotspots).

Table 3. Indicative cost of ELISA analysis. Total cost per sample is dependent upon the number of samples run per plate.

Item	Details	Cost AUD$
P-CTX-1B test kit & refrigerated international shipping	Based on purchase & shipping of 9 test kits in 2022. Bulk purchases may reduce relative shipping costs, but need to consider 6 month shelf-live for test kits Depending on configuration can run 48-58 samples per kit.	$1700 per test kit.
Attophos	Fluorescent detection substrate. Shelf-life of ~ 1 year.	$167 for each test kit
General consumables	Pipette tips, sample vials, gloves, etc.	$100 for each test kit
Labour	Skilled laboratory technician @ $60/hour. Requires a minimum of 4 hours total = 1 h for set up, 2.5h for testing, 0.5 h for reporting.	$270
Approximate total cost per sample	Dependent upon the number of samples run in each plate. ***This does not include labour, equipment or consumables for sample extraction. Current estimates are that extraction takes ~6+ hours and requires a fully equipped chemical analysis laboratory.**	**$40-50 per sample**

10.4 Conclusions

ELISA performance parameters

The ELISA performed within the specifications advertised by the manufacturer when fish extracts resuspended in 80% methanol were diluted 20 times in assay buffer prior to analysis. The ELISA limit of detection was determined to be between 0.5 – 1 x 10^{-3} ng P-CTX-1/mL, which translates to 0.01-0.02 ng P- CTX-1/mL in the original fish extract when considering the required x 20 dilution factor. This LOD in turn translates to a detection level of 0.002 – 0.004 µg P-CTX-1/kg of fish flesh (using 5 g fish tissue extraction). Further analysis of low concentration P-CTX-1 standards and serial diluted positive CTX samples would be required to narrow down the LOD and LOQ.

Across all tested P-CTX-1 standard concentrations (0.94 – 60 x 10^{-3} ng P-CTX-1/mL), the assay yielded acceptable standard recoveries across multiple (n=11) assay runs for both in date test kits and those that had expired 2 months prior to testing. The variation (%CV) for the highest and lowest P-CTX-1 standards between 11 assay runs was less than 15% CV and within manufacturers specifications. Furthermore, the ELISA reliably indicated the presence of P-CTX-1 in 12 different P-CTX-1 positive samples tested in three

independent assay runs conducted on three different days. Significant variability in the estimated P-CTX-1 concentrations of positive samples between assay runs are likely a product of plate drift. For future analysis for quantitative purposes, we recommended reducing the pipetting time of individual samples to avoid the effects of plate drift. Plate drift was not observed to have an impact on whether low P-CTX-1 concentrations were detected or not, suggesting that lengthening the initial incubation time would not improve detection levels any further.

Method comparison & logistics

Where quantifiable levels of P-CTX-1 were detected in fish flesh and livers, all three detection techniques were in good agreement, reporting similar trends in P-CTX-1 concentrations between samples. Both the N2a and ELISA methods indicated the presence of P-CTX-1 in samples where none was detected via LC-MS. The ELISA appeared most sensitive, indicating the presence of ciguatoxin in samples where it was not detected by N2a nor LC-MS analysis. This may be due to the comparatively lower level of detection for this assay (i.e. compare LOD of 0.002-0.0037 µg P-CTX-1 per kg of fish tissue for ELISA and 0.0096-0.17 µg/kg for the N2a and ~0.01 µg/kg for LC-MS (FAO/WHO, 2020). Comparison of the three methods across multiple different samples yielded some interesting observations on potential sample interferences for both LC-MS (CTX not detected while both N2a and ELISA reported high P-CTX-1 levels) and ELISA (indicated high P- CTX-1 concentrations when none were detected with LC-MS nor N2a). Neither of these could be fully explained in this pilot work and future work should consider running tandem assays to provide further insight into the likely causes of these interferences.

The most time limiting step of CTX analysis are the lengthy extraction procedures required prior to analysis (~6.5 h for 10 samples). Following on from this pilot work, it would therefore be of considerable interest to confirm whether crude extracts could be run on the ELISA that do not require the lengthy extraction steps required for LC-MS analysis. This would considerably increase the cost-effectiveness of CTX analysis, opening the possibility to pre-screen samples with the ELISA. Any positive detections of concern could then be followed up with LC-MS and/or N2a if required. Considering the cost of ELISA analysis (~$40-50 per sample for analysis only), such an approach will not be feasible for blanket screening of fish before market. Instead, if employed in a research/environmental monitoring capacity (e.g. identification & monitoring of sentinel reef fish in ciguatera hotspots), it could provide further information on the prevalence of ciguatoxins not only in Spanish Mackerel, but also other economically important reef fisheries that might be impacted.

One of the key factors that limited the extent of this pilot work was the availability of ciguatoxin positive material during the 2021-2022 fishing seasons. Sampling known ciguatera hotspots would increase the likelihood of obtaining further CTX positive material required to further validate the use of CTX analysis (e.g. testing of crude extracts on ELISA). This would provide further certainty on the robustness of different analytical techniques and provide confidence in future data collections that aim to inform refined biotoxin management strategies.

11 Analysis of Spanish Mackerel samples from NSW and QLD for CTXs

11.1 Background

The significant number of CP cases reported since 2014 in Australia (Figure 34, Table 6) generated concern among the commercial and recreational fishing communities, highlighting the need to determine appropriate management strategies to prevent CP illnesses in Australia. In an initial FRDC project 2014-035, a relatively high proportion of a small sample of Spanish Mackerel caught from QLD and NSW waters were found to contain detectable CTXs. In that study, detectable P-CTX-1B was present in both muscle and liver tissues in fish from NSW (n =71, 1.4% prevalence rate, with a confidence interval of 1%–4%, and 7% prevalence, 1%–12%, in flesh and liver, respectively). In the small sample of fish from Queensland, there was a 46% prevalence (19–73%, n=13). Toxin levels found were 0.13 µg kg^{-1} to<0.1 µg kg^{-1} in muscle flesh, and 1.39 µg kg^{-1} to<0.4 µg kg^{-1} in liver, indicating that liver tissue had a significantly higher concentration (~5 fold) of P-CTX-1B. No apparent relationship was observed between the length or weight of *S. commerson* and the detection of P-CTX-1B (Kohli et al 2017). Given the need to understand the distribution and abundance of fish contaminated with CTXs in NSW and QLD, it was determined that samples from two other fishing seasons (2020/2021 and 2021/2022) would need to be collected to have more representative data coverage in order to understand prevalence rates of CTXs in Spanish Mackerel stocks in eastern Australia. Data was also sourced from independent sampling carried out annually by QLD Health on fish associated with CP cases in QLD. With several years of information on CTXs in Spanish Mackerel, it might then be possible to determine environmental, temporal and spatial trends in CTX presence, as well as trends related to fish size or other factors.

The purpose of this chapter was specifically to:

- Generate qualitative and quantitative information about CTXs in Spanish Mackerel (*Scomberomorus commerson*) caught in NSW and QLD waters.

- Investigate CTX presence and concentration concerning factors such as fish size, catch location, date, and environmental conditions.

11.2 Methods

11.2.1 Sample collection

Sampling kits were distributed to fishing clubs and commercial fishing groups in Sydney, QLD and the northern NSW coast. The majority of the Spanish Mackerel catch in NSW is recreational and comes from these areas. The sample pack consisted of several labelled tubes, which could contain ~10g samples of liver and muscle (flesh) tissue. It also contained a laminated diagram explaining the project and how to take samples, a data sheet in order to record information about the fish, and the contact details of the scientists involved. Following sample collection, samples were stored at -20 ˚C until further analysis. The date of catch, length from head to tail and weight of the specimen were recorded. The sampling kit and information sheet is shown in the Appendix, Figure A6.

Fish were collected by individuals from: Coffs Harbour Fishing Cooperative, Ballina Fishing

Cooperative, Byron Bay Deep Sea Fishing Club, Mackay Game Fishing Club, Newcastle Neptune's Spearfishing Club, Tweed-Gold Coast Freedivers Club, the Sydney Fish Market, and the NSW Department of Primary Industries Research Angler Program.

Additional information regarding CTX positive samples from QLD was sourced from the QLD Health. QLD Health provided information on location, size and CTX content (P-CTX-1B, 52-epi-54- deoxy-CTX-1B (formerly known as CTX-2) and 54-deoxy-CTX-1B (formerly known as CTX-3) of the collected Spanish Mackerel specimens. Toxins were analysed using LC-MS by QLD Health.

11.2.2 Fish sample extraction

Each tissue sample was chopped using a scalpel blade and 5 ± 0.1g biomass was weighed, and placed in a 50 mL centrifuge tube. To this, 15 mL of 60 % LC-MS grade Methanol (Sigma, St. Louis, MO) was added and the tissue samples were homogenized using an Ultra-Turrax (Thermo Fisher, Waltham, MA) at maximum speed for 1 min. The tissue samples were then incubated at 95 ˚C for 10 min and cooled on ice for 5 min. Further, tissue samples were centrifuged at 3200 x g for 10 min to pellet insoluble debris and a 5 mL aliquot of the supernatant was transferred to a new 15 mL centrifuge tube for liquid-liquid partitioning.

11.2.2.1 Liquid-Liquid Partitioning

A 5 mL aliquot of LC-MS grade dichloromethane (DCM) (Sigma, St. Louis, MO) was added to the 5 mL of sample extract and then vortexed for 15 seconds. Samples were centrifuged at 3200 x g for 1 min to ensure partitioning of the solvent layers and the volume in the top layer (aqueous methanol) was aspirated, and the lower DCM layer was aspirated down to 4 mL level. The remaining 4 mL of DCM-toxin mix was taken to dryness in a 55˚C heating block and under a nitrogen flow.

11.2.2.2 Solid Phase Extraction

A 200 mg/3mL solid phase extraction cartridge CUNAX123 (United Chemical Technologies, Levittown PA) was conditioned with 10 mL DCM. The dry sample-residue was dissolved in 4mL DCM and the entire volume loaded onto the cartridge. The cartridge was washed with 4 mL DCM. For elution, 4 mL of 9:1 dichloromethane:methanol was passed through the cartridge and the volume collected in 10 mL tubes. Further, the samples were taken to dryness at 55˚C under a stream of nitrogen. The dry sample tubes were stored at -80˚C until LC-MS analysis. For analysis, the dried samples were reconstituted in 200 µL of 80% methanol and transferred into a glass autosampler vial.

11.2.3 Liquid Chromatography-Mass Spectrometry Analysis

Analysis of the fish extracts was performed at SIMS in Sydney using a high resolution LC-MS system and the Cawthron Institute in New Zealand using a triple quadrupole LC-MS/MS instrument.

With both instruments chromatographic separation used a Waters® Acquity UPLC BEH Phenyl (1.7 µm, 100 x 2.1 mm column) column held at 50°C. The mobile phases consisted of (A) Milli-Q containing 0.2% ammonia and (B) Acetonitrile containing 0.2% ammonia. Each buffer solution was prepared freshly every day. The gradient conditions are described below (Table 4).

Table 4. Gradient conditions used during LC-MS analysis.

Time [min]	A [%]	B [%]	Flow [µL/min]
0.00	60.0	40.0	550
2.00	40.0	60.0	550

2.50	5.0	95.0	550
3.00	5.0	95.0	550
3.01	60.0	40.0	550
5.00	60.0	40.0	550

At Cawthron the analysis was performed on a Waters Xevo TQ-S triple quadrupole mass spectrometer coupled to a Waters Acquity UPLC i-Class with flow through needle sample manager. An injection volume of 2 µL was used. The electrospray ionization source was operated in positive-ion mode at 150 °C, capillary 3.5 kV, cone 30 - 75 V, nitrogen gas desolvation 1000 L h^{-1} (600 °C), cone gas 150 L h^{-1}, and the collision cell argon gas flow 0.15 mL min^{-1}. For quantitative analysis, a total ion chromatogram generated from the following multiple reaction monitoring (MRM) transitions was used: m/z 1128.6>95.0 (CE 65 eV), m/z 1128.6>109.0 (CE 55 eV) m/z 1133.6>1133.6 (CE 55 eV). A dwell time of 20 ms was used for all transitions monitored. Peak areas were integrated and sample concentrations calculated from linear calibration curves generated from standards. TargetLynx software was used for the analysis (Water- Micromass, Manchester, UK).

11.2.4 Spike Recovery

To ensure satisfactory performance of the method, numerous flesh and liver samples were analysed in duplicate, with one of the samples spiked with a known amount of P-CTX-1B standard (11 of 168 samples). The spiking of samples with CTX was for calibration purposes only, and these results were not included in the final concentrations. Mean recoveries were calculated for each matrix and applied to the toxin concentration determined in samples. The P-CTX-1B spiking solution was provided by the Cawthron Institute in Nelson, New Zealand with a given concentration of 58.651 ng/mL. Additionally, for instrument calibration the Cawthron Institute provided three standard solutions with the P-CTX-1B-concentrations of 0.341 ng/mL, 1.705 ng/mL & 3.41 ng/mL. These calibration standards were analysed at the same time as the various fish samples and were used to create a calibration curve. The concentration of P-CTX-1B was calculated by comparing the peak areas observed in contaminated fish samples with the calibration curve generated at the time of analysis.

11.2.5 Spanish Mackerel identification via qPCR

To determine the identity of fish specimen collected DNA was extracted from approx 20 mg of flesh from fish specimens using QIAamp 96 DNA Qiacube HT Kit (Qiagen) . Flesh samples were incubated in proteinase K and lysis buffer provided by the manufacturer. The lysate was then purified using wash buffers as per manufacturer's instructions. DNA was quantified using Nanodrop ND-1000 spectrophotometer and analysed using the qPCR primers (Forward: TGGGCCGTCCTTATTACAGC, Reverse: CTCCTCCTGCTGGGTCAAAG) specific for the cytochrome oxidase subunit I (COI) gene from *S. commerson* (Ward et al., 2005).

Table 5. Cycling conditions used for qPCR identification of *S.commerson* specimens**.**

Step	**Temperature**	**Time**
Holding stage	95 ˚C	10min
Cycles	95 ˚C	15s
	60 ˚C	1min
Melt curve	95 ˚C	15s
	60 ˚C	1min
	95 ˚C	30s

All PCR reactions were performed in 5 µL reaction volumes containing 2.5 µL iTaq Universal SYBR Green Supermix (Biorad), 1.1 µL nuclease free water, 0.2 µL of forward and reverse primer (0.5 µM final concentration) and 1 µL of DNA template. The plate was prepared with an epMotion®5075l Automated Liquid Handling System . The qPCR assay was performed using the BIORAD CFX384 Touch™ Real-Time PCR Detection System™ using a 95 °C holding stage for 10 min, followed by 35 cycles of 95°C for 15 s and 60°C for 1 min, followed by a melt curve analysis (Table 5, Figure A7). Spanish Mackerel from previous studies (FRDC project 2014-035) was used as a positive control and Purple Rock Cod (*Epinephelus cyanopodus*) was used as a negative control for this analysis. All samples were verified based on having similar melt curves and amplification cycles to the positive control.

11.3 Results and Discussion

11.3.1 Spanish Mackerel from fishing seasons 2014-15, 2020-21 and 2021-22

Samples of Spanish Mackerel were collected in NSW and QLD in the 3 fishing seasons, 2014-15, 2020-21 and 2021-22. All samples were verified to be Spanish Mackerel via qPCR analyses.

During the 2014-15 fishing season, a total of 84 samples were collected and analysed for CTXs (Table A8). Using LC-MS analysis, P-CTX1B was detected in 5 fish specimens from NSW (Table A8). Among the 13 fish specimens collected in QLD, P-CTX1B was found in the liver and flesh tissues of six different fishes.

For the 2020-21 fishing season, 101 fish were collected and analysed for CTXs. Fish were from 2.7-21.8 kg in size, and collected from locations in northern NSW and QLD. P-CTX-1B was below the limit of detection (LOD) for all flesh and liver samples analysed via LC-MS (Table A10).

For the 2021-22 fishing season, 148 fish were collected and analysed for CTXs. Fish were from 2.8-21.5 kg in size, and collected from locations in northern NSW and QLD. P-CTX-1B was below the limit of detection (LOD) for all flesh and liver samples analysed via LC-MS (Table A9).

During the course of researching Chapter 10, it was determined that the ELISA test kit was more sensitive with a lower LOD than the LC-MS method for the measurement of CTX-1B. Hence, it was decided to verify they lack of CTXs in specimens by analysing them using the ELISA CTX method. The 148 specimens from the 2021-22 fishing season were analysed as described in the Methods of Chapter 10. P-CTX-1B amounts were detected in 18 flesh and 14 liver samples (35 fish of 148) but were generally below the limit of quantification for the ELISA test kit (Table A9). Three samples from the fishing season 2021-22 exceeded the recommended ≥ 0.01 ug/kg P-CTX-1 B equivalents set by the U.S. Food and Drug Administration (FDA) as a guidance level for CTXs in seafood. The highest level was found at 0.012 µg/kg (Table A9).

Fish caught in QLD were considerably more likely to contain CTXs than fish caught in NSW over the three fishing seasons, based on data from LC-MS for the 2014-15 samples and data from the ELISA method for the 2021-22 samples. Based on the data from the ELISA method, in the 2021/22 fishing season, no fish analysed in the study that was caught in NSW waters (0 of 32) were found to contain CTXs, whereas 35 of 116 fish (30%) from QLD were found to contain some CTXs, usually below the level of quantification (Table A9). These CTX+ fish were collected from the vicinity of Fraser Island, Hervey Bay, Rockhampton, Wigton Islands and Coolum.

A known ciguatoxic Spanish mackerel was extracted periodically alongside the environmental samples and showed consistent detections for P-CTX-1B, despite the low level of CTX and large variability (Tables A1 and A2). Full spike results showed a comparatively low recovery of P-CTX-1B from tissue samples across both seasons, which was lower than what has been historically observed using the extraction protocol (Table A3). The extraction of CTXs from fish matrix tissue presents unique challenges, with extraction efficiencies observed to be comparatively low and variable in our study. This is in concordance with what has been previously observed in other studies with Spanish Mackerel of general fish tissue samples spiked with P-CTX-1B prior to extraction, that have reported recovery rates of 25.8% (Kohli et al. 2017), 44% (Murray et al. 2018), and 24-110% (Spielmeyer et al. 2021). Unlike other marine biotoxins and shellfish matrices, CTX extraction from fish tissue is generally less efficient. These results underscore the necessity for further research and optimization of extraction methods to enhance detection and quantification of CTXs in fish samples.

To ensure confidence in the non-detects for the environmental samples, 16 fish were selected based on their length, weight and geographical location and were re-extracted a second time at the Cawthron Institute. All samples were again blank giving confidence that the extraction protocol was not a significant factor in the ability to recover CTXs.

11.3.2 Analysis of samples from QLD Health and statistical analyses

Nineteen outbreaks of CP were reported to QLD Health over the period 2019- 2023 (Figure 28, Figure 34). Of these, information on the size and weight of Spanish Mackerel associated with these outbreaks was collected, and P-CTX 1B was measured using LC-MS. These data were added to our dataset from fishing season 2014-15 to examine the relationship of fish size with CTXs.

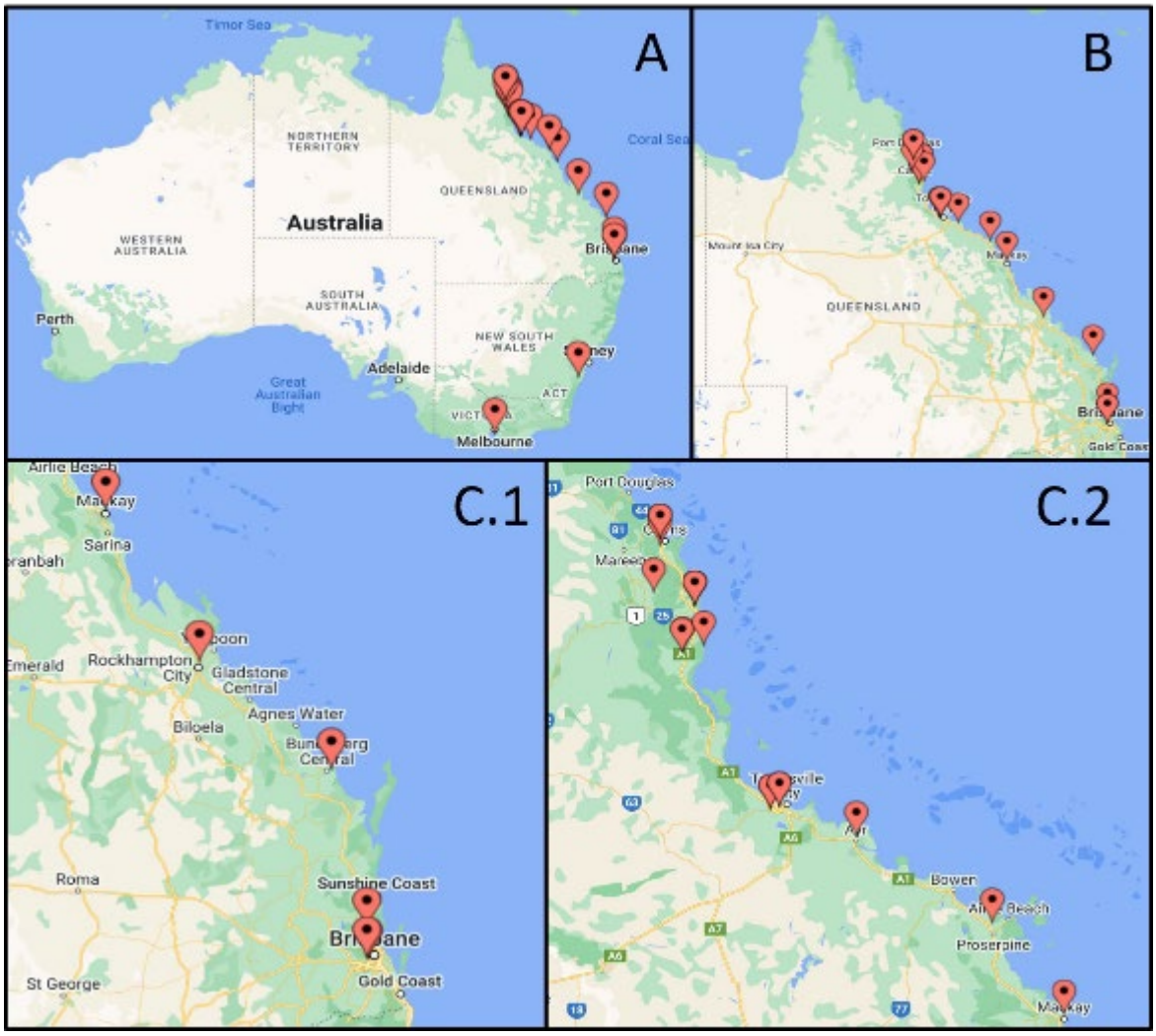


Figure 28. Map highlighting the sites where CP cases occurred over the period 2019-2023, in information provided by QLD Health (A). Focus on Queensland (B). Brisbane to Mackay (C.1) and Mackay to Port Douglas (C.2).

No significant correlation was observed between the amount of P-CTX-1B and the weight of the fish (Figure 29). Despite the absence of a statistical correlation, a higher number of fishes below 15 kg showed the presence of CTXs rather than the larger specimens, an observation that aligns to research conducted in French Polynesia on other fish species (Gaboriau et al., 2014).

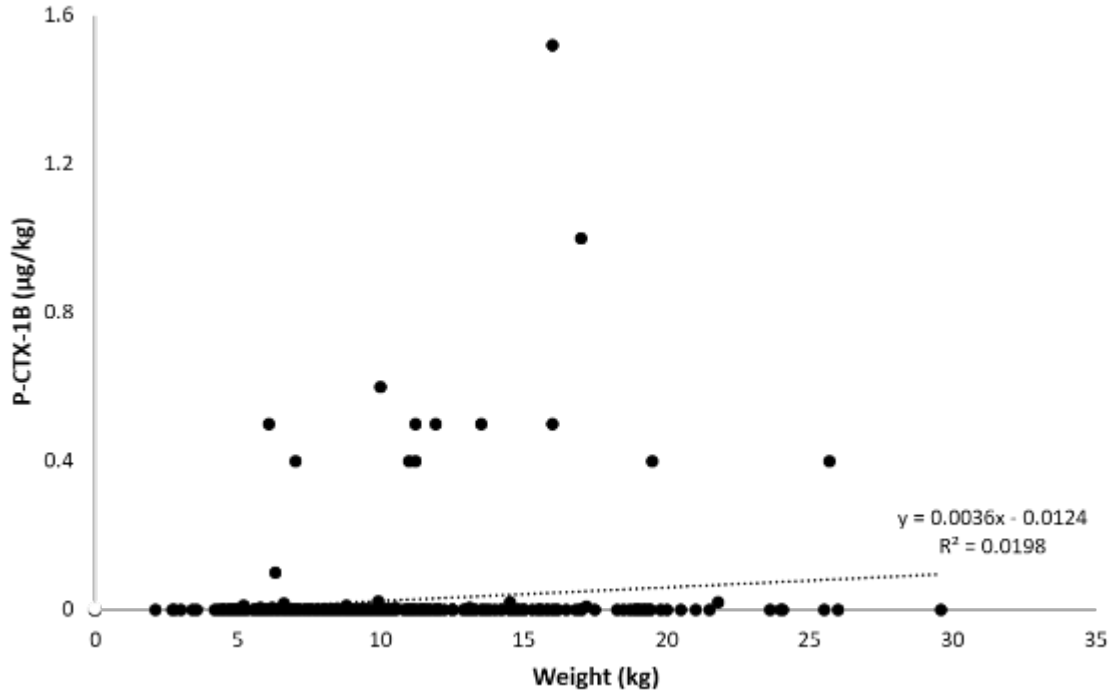


Figure 29. Fish weight (kg) and CTX content using LC-MS (µg/kg) of all Spanish Mackerel samples collected (2015-2022).

To further explore the possible relationship between fish size and CTX contamination, physical data from fish samples that tested positive and negative for CTXs were combined and plotted together (Figure 30). The graph clearly shows that as the length of the fish increases, its weight also increases exponentially (as observed for Onespot Snapper (Figure A1), Flowery Rockcod (Figure A2), Red Bass (Figure A3) and Yellowedge Coronation Trout (Figure A4)). However, there is no direct evidence to suggest that fish below a certain weight are more likely to contain CTXs, similarly to what observed by Oshiro et al., (2010) (Figures A1-A4). Among the 25 positive samples of our study, 14 had a weight below 15 kg and a length below 120 cm. Additionally, the relationship between weight and length appears stronger in positive samples (R^2= 0.92), but further analyses of more positive fishes are needed to confirm this observation.

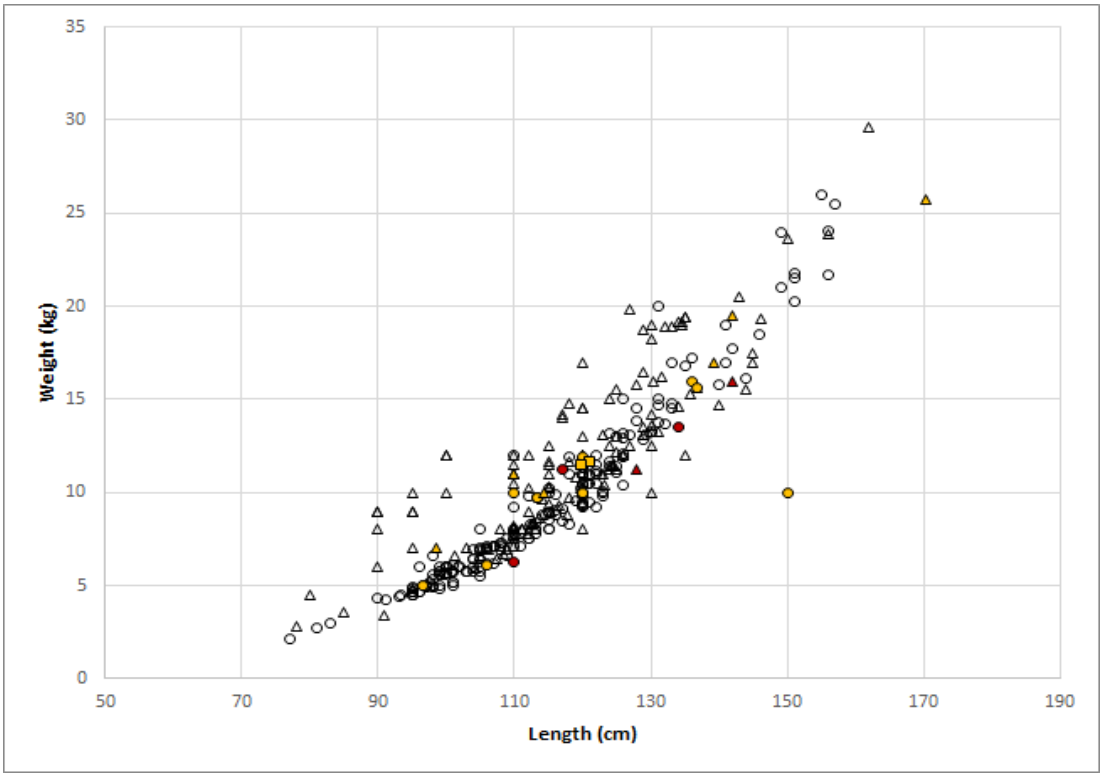


Figure 1. Relationship between weight (kg) and length (cm) of fish samples found negative (clear circles and triangles) and positive (red or yellow circles or triangles) for CTXs, as measured using LC-MS. Samples from NSW are shown as triangles, while those from QLD are shown as circles. Yellow dots and circles indicate those fish shown to be positive for CTXs using LC-MS (n=16). Red circles or triangles indicate those samples shown to be positive using the ELISA kit described in Chapter 10 (n=5). Sample were collected (2015-2023).

CTX content in relation to the weight was analysed, organized by the state where the samples were collected. No statistical correlation can be observed (Figure 31).

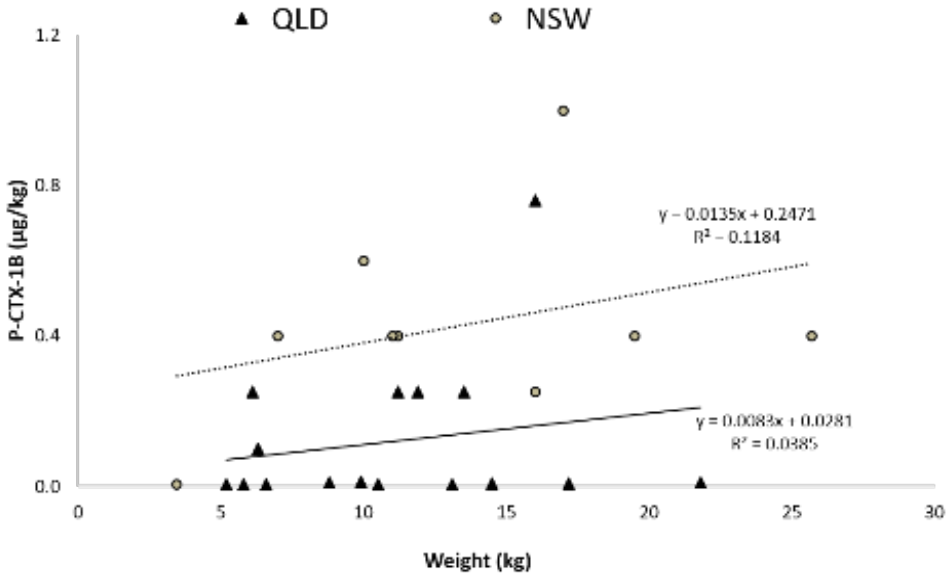


Figure 31. CTX content in Spanish Mackerel according to the weight and geographical location in which they were collected. Sample collected between 2015-2022. Dotted line represents NSW trend, black line represents QLD trend.

Table 6. List of confirmed CP cases caused by consuming fish caught from NSW waters.

Date	Cases	Fish Species/Origin	P-CTX-1B (μg kg^{-1})
Feb. 2014	4	Spanish Mackerel/Evans Head, NSW	nd, 0.6, 1
Mar. 2014	9	Spanish Mackerel/Scotts Head, NSW	0.4
Apr. 2015	4	Spanish Mackerel/South West Rocks, NSW	n/a
Mar. 2016	3	Spanish Mackerel/Crowdy Head, NSW	0.93
Apr. 2016	4	Spanish Mackerel/Crescent Head, NSW	0.11, 0.37
Feb. 2018	4	Spanish Mackerel/Coffs Harbour, NSW	n/a - no samples available
Apr. 2018	3	Spanish Mackerel/Wooli, NSW	n/a - no samples available

Fish caught in QLD, particularly in the Fraser inshore region and Hervey Bay, have been linked to CP. These areas are within the Great Sandy Marine Park and include Platypus Bay, where CP has been well-documented since the late 1970s and 1980s. The region boasts extensive seagrass meadows, and Spanish Mackerel, Barracuda, and Blotched-javelin caught here have all been associated with CP.

Spanish Mackerel are the largest mackerel species in Australian waters, known for their size, taste, and the excitement of catching them. While they can reach lengths of up to 2.4 m and weights of up to 70 kg, such large specimens are now rarely caught. The largest recorded catch in recent years was a 54-kg fish off Fraser Island in 2015. Interestingly, data from the three fish responsible for CP intoxication revealed that fish of varying weights can carry different amounts of CTXs (0.6, 1 and 0.4 μg/kg, as shown in Table 6). These specific fish weighed 10, 17, and 25 kg (Table A8), with the largest fish having the lowest level of CTXs. These findings again suggest that there is no clear correlation between fish weight and CTX concentration.

Historically, most CP cases along the east coast of Australia have been associated with Spanish Mackerel caught south of approximately Mackay (around 21°S latitude). However, there have been no new reports of CP in NSW since 2018. This information parallels our finding of comparatively little or no CTXs in the Spanish Mackerel collected in our 2020-2021 and 2021-2022 fishing seasons with LC-MS, which was notably lower than was found in 2014-2016. Potential environmental factors associated with CTXs in QLD and NSW are reviewed in the following section.

11.3.3 Effects of Natural Disturbances on Spanish Mackerel CTX Content

Several studies have connected natural disturbances such as cyclones with increased cases of CP, as reported in Rongo & van Woesik, (2013). In the same study the authors noticed a relationship between the increase of CP cases and the increase of severity of disturbance. This correlation coincided also with the inter-annual cycle of El Niño Southern Oscillation (ENSO).

It appears that the substantial waves generated by cyclones have the effect of resetting the pattern of algal succession (Rongo & van Woesik 2013). This, in turn, creates favourable conditions for the establishment of ciguatoxic dinoflagellates, Consequently, this phenomenon raises the likelihood of CP. For instance, cyclones can mix and upwell ocean waters, bringing nutrients from deeper layers to the surface. This increased nutrient availability can promote the growth of phytoplankton, including *Gambierdiscus*, and led ultimately to an increase of algal blooms. Moreover, previous studies have proposed that early-successional, opportunistic turf algae (such as *Gambierdiscus* spp.), in comparison to late-successional algae, are characterized by higher nutrient content and enhanced palatability (as observed in Littler & Littler, 1980, and Steneck & Dethier, 1994). In the Cook Islands, after the cyclones of 2003-2005, there was a notable increase in the prevalence of these opportunistic turf algae, which play a significant role as hosts for ciguatoxic dinoflagellates, as documented in Cruz-Rivera and Villareal, 2006. This increase heightened the potential for the transfer of CTXs into the food web through herbivorous fish.

The 2014-15 cyclone season in northern Australia was below average but unusually intense: only seven cyclones affected the Australian region during the season (November-April), but almost all belonged to category three, four or five (Table 7). In the Australian region, this was the first season in the last 35 years where every cyclone, regardless of whether they made landfall or not, attained the status of severe tropical cyclones, according to the BOM climatologist Joel Lisbonbee (https://www.9news.com.au/national/australia-s-strange-2014-15-cyclone-season/05b40d95-a193-4ca9-8533-7953bdfee6af, Figure 32). On the other hand, in the 2021-2022 cyclone season only two out of ten were categorized as severe BOM reports, http://www.bom.gov.au/, Figure 32). These climatic events could be associated with the higher proportion of CTXs and greater number of CP cases observed in the 2016 peak of CP cases. However, it's worth noting that the low disturbance frequency observed in the 2021-22 season could potentially increase the probability of CP events. These changes in cyclone patterns can trigger a series of societal and ecological consequences. A fear of CP can lead to a decline in fishing activities (Rongo and van Woesik, 2013; Chinain et al., 2023), which, in turn, results in an increase in fish populations and a decrease in reported CP cases. This, paradoxically, fosters the belief that reef fish are safe to consume, potentially leading to overfishing and can elevate the risk of CP.

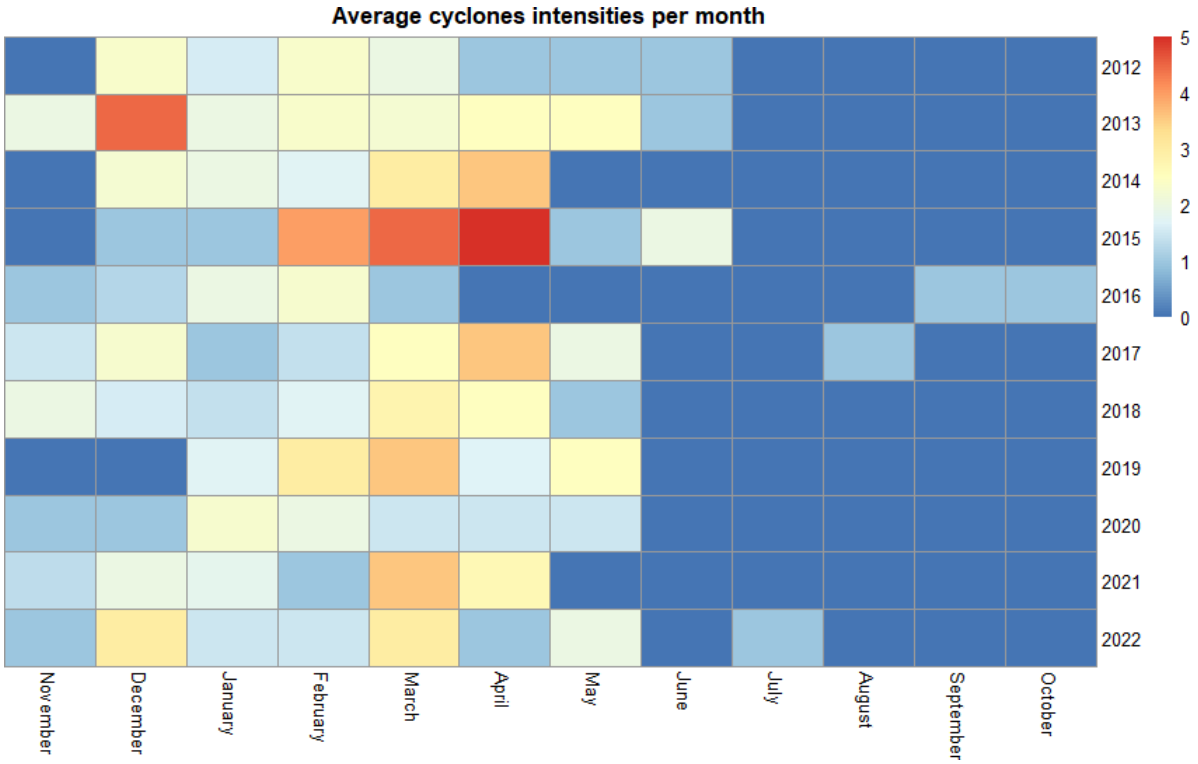


Figure 32. Average cyclone intensities per month from 2012 to 2022. Intensity 0 corresponds to undetected activity, 0-1 to Depression (wind between 31–50 km/h*), 1-2 to Deep Depression (wind between 51-62 km/h*) , 2-3 to Cyclonic Storm (wind between 63-88 km/h), 3-4 to Severe Cyclonic Storm (wind between 89-117 km/h*), 4-5 to Very Severe Cyclonic storms (wind between 118-165 km/h*), 5 to Extremely Severe Cyclonic Storm (wind between 166-220 km/h*), above 5 to Super Cyclonic Storm (wind more than 220km/h*). *3 min average measurements.

A positive correlation between SOI (southern oscillation index), as well as El Niño or La Niña events and CPUE (catch-per-unit-effort) for Spanish Mackerel has been previously observed, with higher catches during La Niña events and lower during El Niño (Welch et al., 2014, for more information see paragraph 9.5.2). The current condition of the ENSO is the neutral state, which is neither El Niño nor La Niña, and has persisted over the past three years and may lead to an increase of CP (2019-2022). During this period, Spanish Mackerel fishing declined, accompanied by a decrease in CP reports and CTX levels in the individual fish caught. Moreover, the current neutral state, with prevailing winds carry warm, moist air and warmer surface waters towards the western Pacific, provides an ideal environment for the proliferation of *Gambierdiscus* species. Consequently, an increase in CTX content in fish and the potential for ciguatera poisoning outbreaks remain significant concerns. Therefore, sampling not only Spanish Mackerel but also *Gambierdiscus* species in known CP hotspots is likely to yield positive material for CTXs, to validate the use of different strategies to detect them. A more extensive sampling approach will provide insights that contribute to a better understanding of CP, knowledge that can be used to define monitoring strategies.

Table 7. Locations impacted by cyclonic disturbances and the number of such disturbances during the years 2012-2015 (http://www.bom.gov.au).

Place affected	Cyclonic Storm	Severe Cyclonic Storm	Very Severe Cyclonic Storm	Extremely Severe Cyclonic Storm
Cape York Peninsula	1	-	-	-
East Timor	-	-	1	1

Indonesia	1	-	-	1
New Caledonia	-	1	-	-
New Zealand	-	-	-	1
Northern Territory	1	-	-	1
Papua New Guinea	-	-	-	1
Queensland	1	1	2	3
Solomon Islands	-	1	-	1
South Australia	-	-	1	-
Tonga	1	-	-	-
Western Australia	2	4	3	-

12 Discussion

Food safety risks in Australia and New Zealand are managed under a joint food regulatory system. Core elements of that system are model legislation described as “model food provisions” and food production and labelling standards named the “Australia New Zealand Food Standards Code” (Code). The model food provisions and the Code have been adopted by each Australian state and territory as the basis for their respective food legislation. (Australian Food Regulation Secretariat)

Food Standards Australia New Zealand (FSANZ), a statutory authority in the Australian government health portfolio, maintains the Code, subject to policy set by the Australia and New Zealand Ministerial Forum on Food Regulation, to ensure that food is safe and suitable for human consumption. In Australia, the model food provisions and the Code are enforced domestically by state and territory departments, agencies and local councils. In addition, the Australian federal government Department of Agriculture, Fisheries and Forestry (DAFF) enforces imported food compliance with the Code. Within NSW, the NSW Food Authority is the relevant domestic regulator. The relevant NSW legislation is the *Food Act 2003* (NSW), the Food Regulation 2015 (NSW) and the Code. This includes a general requirement under the Food Act to ensure food supplied is both safe and suitable (ss 16 and 17) and specific requirements for managing seafood safety risks through a Seafood Safety Scheme under Part 11 of the Food Regulation 2015 (NSW).

CP risk is highly complex and management of CP requires a multifaceted approach that traverses environmental, food safety and health variables. A flow diagram (Figure 33) that summarises current CP responses and needs (FAO and WHO, 2020) highlights the many intricate subjects involved in understanding and managing CP. The current status of CP management and regulation in NSW, and the rest of Australia, reflects the limitations and knowledge gaps of this syndrome. Within the Food Standards Code, Schedule 19 *Maximum levels of contaminants and natural toxicants*, provides maximum limits for algal toxins such as paralytic shellfish toxins, diarrhetic shellfish toxins and amnesic shellfish toxins (FSANZ, 2023). There is no equivalent maximum concentration limit for CTXs in seafood in the Food Standards Code. This is primarily due to testing limitations and limited reference standard availability. In addition, in Australia the position has been that risk is dependent on the size and type of fish consumed. As a result, in lieu of testing, management approaches to CP are precautionary with fishing bans and restrictions on locations and fish sizes for known ‘hot spots’. The 2006 Guide to the Australian Primary Production and Processing Standard for Seafood developed by FSANZ (FSANZ, 2006), notes that CTXs are a potential hazard and provides similar advice to skippers to avoid fishing in areas that are known to be linked to CP outbreak and/or be aware of size restrictions on certain fish species. This aligns with the general principle, that food contaminants should be as low as reasonably achievable regardless of whether maximum limits are established (FSANZ, 2006).

Such measures and guidelines are in place at Sydney Fish Market (Sydney Fish Market, 2015) to safeguard consumers against CP. For example, Platypus Bay, QLD is a prohibited supply region for Spanish Mackerel and size restrictions (10 kgs whole or 8 kg for headed and gutted fish) are in place for Spanish Mackerel caught from other QLD locations and NSW waters.

Current advice for consumers is published on the NSW Food Authority website: https://www.foodauthority.nsw.gov.au/consumer/food-poisoning/fish-ciguatera-poisoning

Figure 33. Flow diagram showing ciguatera poisoning responses and needs (from FAO and WHO, 2020).

12.1 Risk assessment based on project data

Risk assessments for food contamination consists of four formal science-based steps: hazard identification, hazard characterisation, exposure assessment and risk characterisation (FAO and WHO, 2023). Table 8 discusses these steps in the context of the available information and the results of this project.

Table 8. Summary of risk assessment process (FAO and WHO, 2023) within the context of the current project.

Risk Assessment Process	**Process Definition**	**Status**
1. Hazard identification	The identification of biological, chemical, and physical agents capable of causing adverse health effects and which may be present in a particular food or group of foods.	• CTXs are highly potent neurotoxins that can bioaccumulate and biotransform in the marine food chain. Human illness occurs when contaminated seafood is consumed. • Some of the highest risk fish are predatory species from warm water, tropical areas. • Currently there is no valid method of establishing whether a specific 'catch' from a high risk area does or doesn't pose a CP risk.
2. Hazard characterisation	The qualitative and/or quantitative evaluation of the nature of the adverse health effects associated with biological, chemical, and physical agents which may be present in food.	• CTXs cause a range of gastrointestinal, neurological and cardiovascular symptoms, with a complex array of clinical manifestations. • In humans, the individual response to ciguatoxin exposure can vary, with potential for chronic and recurring issues. This is also related to portion size (dose) and previous exposure to ciguatoxins. • P-CTX-1 is the most potent of known ciguatoxins, but information is limited, and we do not yet understand how the other (more than 30) analogues contribute to illness. • CP cases linked to Spanish Mackerel caught in NSW waters appeared to spike between 2014 and 2018, with no previous reports since 2002. Since 2018, there have been no confirmed cases of CP linked to Spanish Mackerel caught in NSW waters. The reason for this is not clear, and may be related to environmental variables, fisher awareness or a combination of both. • The nature and extent of patient reporting and clinical diagnosis of cases of CP is unknown but is believed to be poor.

Risk Assessment Process	Process Definition	Status
3. Exposure assessment	The qualitative and/or quantitative evaluation of the likely intake of biological, chemical, and physical agents via food as well as exposures from other sources if relevant.	• CTX levels can vary between individual fish, and tend to be more concentrated in the head, roe, liver or other viscera. The metabolic processes of ciguatoxins are complex. Different fish may metabolise toxins differently (Ikehara et al., 2017). • The previous FRDC Project 2014-035 Safeguarding Commercial Fishing in NSW From Ciguatera Fish Poisoning and the current FRDC Project 2019-060 The Detection of Ciguatera Toxins in NSW Spanish Mackerel determined levels of the ciguatoxin analogue P-CTX-1 (also known as P-CTX-1B) via LC-MS, ELISA and N2a assays in Spanish Mackerel. These baseline data are some of the most extensive Australian data collected in terms of the number of Spanish Mackerel tested and in terms of the timeframes over which the studies occurred (2015 and 2021-2022). P-CTX- 1B results were reported between 0.005-0.43 ng/ml (ELISA), 0.02-0.14 ng/g (N2a) and 0.023-0.063 ng/ml (LC-MS). Samples of the cooked meal or associated fish are not always available during illness investigations. Spanish Mackerel samples linked to CP in NSW reported between 'not detected' and 1 µg/kg P-CTX-1B (Table 6, Chapter 11). This is up to two orders of magnitude higher than the USFDA guidance level of 0.01 µg/kg P-CTX-1B, which is the same level that the European Food Safety Authority's panel on Contaminants in the Food Chain estimate should not have any negative health impacts. • Routine testing of seafood for ciguatoxins has been limited by reference standard availability. A concentration of 0.02 ug/kg CTX1B-equiv is the lowest reported level of ciguatoxins in fish associated with symptoms in humans, but the insufficient amount of animal and human exposure data has limited the establishment of an acute reference dose (FAO and WHO, 2020). • In NSW the food consumed by one reported CP case was analysed and found not to contain P-CTX-1, despite strong clinical symptoms, indicating there are limitations in using current analysis methods to quantify exposure to CP. •

Risk Assessment Process	Process Definition	Status
4. Risk characterisation	The qualitative and/or quantitative estimation, including attendant uncertainties, of the probability of occurrence and severity of known or potential adverse health effects in a given population based on hazard identification, hazard characterization and exposure assessment.	• Risk characterisation is limited beyond current guidelines. • As the level of CTXs can be highly variable between each fish, and fish of different species, it is difficult to extrapolate beyond the specific ciguatoxin analogue tested in each individual fish. • The size of Spanish Mackerel does not seem to be linked to toxin level, but most reported illness cases in NSW were linked to larger (>10 kg) Spanish Mackerel. • Modelling of environmental data may provide insight over longer term studies (e.g., temperature, cyclones, southern EAC intensification). While environmental data may indicate 'hot spot' reefs, Spanish Mackerel are a migratory species, their origin is not easily distinguished, and they can travel several 100 kms. • CP cases are largely underreported (Figure 34), and this has limited our understanding of illness prevalence. Nationally consistent collection and reporting of epidemiological data and linking to toxicological data/case information was identified as a critical issue by the National Ciguatera Strategy (Beatty et al., 2019).

From the literature and our own data, we have compiled information on the P-CTX-1B levels in any fish known to be associated with CP illnesses in Australia (Table 9) and overseas (Table 10). This shows that levels above ~0.1 µg kg^{-1} have been known to be associated with illness, with mean levels found in implicated fish flesh of 1.2 µg kg^{-1} (from 6 Australian samples) and 1.3 µg kg^{-1} (from 16 overseas samples) (Tables 9 and 10). This compares to the US FDA 'guidance level' of 0.01 µg kg^{-1}, which was established due to the consideration that levels above 0.1 µg kg^{-1} may cause illness, based on the results of the mouse bioassay (Lewis et al., 1991). There are several other factors aside from the levels of P-CTX-1B that may lead to differences in toxicity among samples. These are the fact that other CTX analogs likely exist in these fish alongside P-CTX-1B, which we currently cannot measure accurately using LC-MS, as we lack standards for these analogs. The presence of these additional analogs may increase the overall toxicity at low levels of P-CTX-1B. As several of the fish in this study were found to contain P-CTX-1B at very low levels, it appears that further research is required to determine the appropriate safe level of P-CTX-1B in fish in Australia. In any study such as this, it would be necessary to compare fish using several methods, such as toxicity assays (bioassays, or other assays such as the receptor binding assay) as well as by LC-MS/MS

Table 9. P-CTX-1B levels in fish known to be associated with illness with CP symptoms in Australia.

Location	Fish species	P-CTX-1B in flesh (µg kg^{-1})	Reference
Capel Banks, Coral Sea	Purple rock cod	0.1	SIMs Unpublished data
Scotts Head, NSW	Spanish Mackerel	0.4	(Farrell et al., 2016)
Evans Head, NSW	Spanish Mackerel	0.6-1.0	(Farrell et al., 2016)
Capel Bank Seamount	Redthroat Emperor	0.023	(Farrell et al., 2016)
Capel Bank Seamount	Purple rock cod	0.069	(Farrell et al., 2016)
Capel Bank Seamount	Green Jobfish	0.006-0.036	(Farrell et al., 2016)
Crowdy Head, NSW	Spanish Mackerel	0.93	(Farrell et al., 2016)
Crowdy Head, NSW	Spanish Mackerel	0.11-0.37	(Farrell et al., 2016)
Gove, Arnhem Land, NT	Coral Cod	3.9	(Lucas et al., 1997)
Queensland	Sawtooth Barracuda	1.1	(Hamilton et al., 2010)

Table 10. Toxicity and level of P-CTX1B in leftover meals from CP incidents in Japan (Oshiro et al., 2010). 1 MU toxicity equals 7 ng of P-CTX-1B in fish flesh (Yasumoto, 2005).

number of CP cases associated with this outbreak (in Japan)	Fish Species	Test Sample	Mouse Bioassay Toxicity (MU/g)	P-CTX-1B (μg kg^{-1})
2	*Lutjanus sp.,* (Snapper)	Cooked flesh	0.29	2.03
4	*Variola louti* (Yellow-edged Coronation Trout)	Raw flesh	0.1	0.7
13	*Epinephelus fuscoguttatus* (Flowery Rockcod)	Cooked flesh	0.05	0.25
		Soup[1]	<0.025	0.175
17	*Lutjanus monostigma* (Onespot Snapper)	Cooked flesh	>0.2	1.4
20	*Lutjanus monostigma* (Onespot Snapper)	Cooked flesh	>0.8	5.6
22	*Lutjanus monostigma* (Onespot Snapper)	Raw flesh	>0.2	1.4
		Mixed soup[2]	0.025	0.175
23	*Lutjanus monostigma* (Onespot Snapper)	Mixed soup[2]	>0.2	1.4
24	*Variola louti* (Yellowedge Coronation Trout)	Raw flesh	0.4	2.8
		Mixed soup[2]	0.1	0.7
26	*Variola louti* (Yellowedge Coronation Trout)	Flesh[3]	>0.2	1.4
26	*Variola louti* (Yellow-edged lyretail)	Flesh[3]	0.1	0.7
28	*Variola louti* (Yellowedge Coronation Trout)	Raw flesh	0.1	0.7
31	*Lutjanus bohar* (Red Bass)	Cooked flesh	0.1	0.7
32	*Variola louti* (Yellowedge Coronation Trout)	Raw flesh	0.05	0.35

[1]Assay was performed after removing flesh and bones present in the soup.
[2]Assay was performed after removing bones present in the soup.
[3]The flesh had been lightly rinsed with hot water.

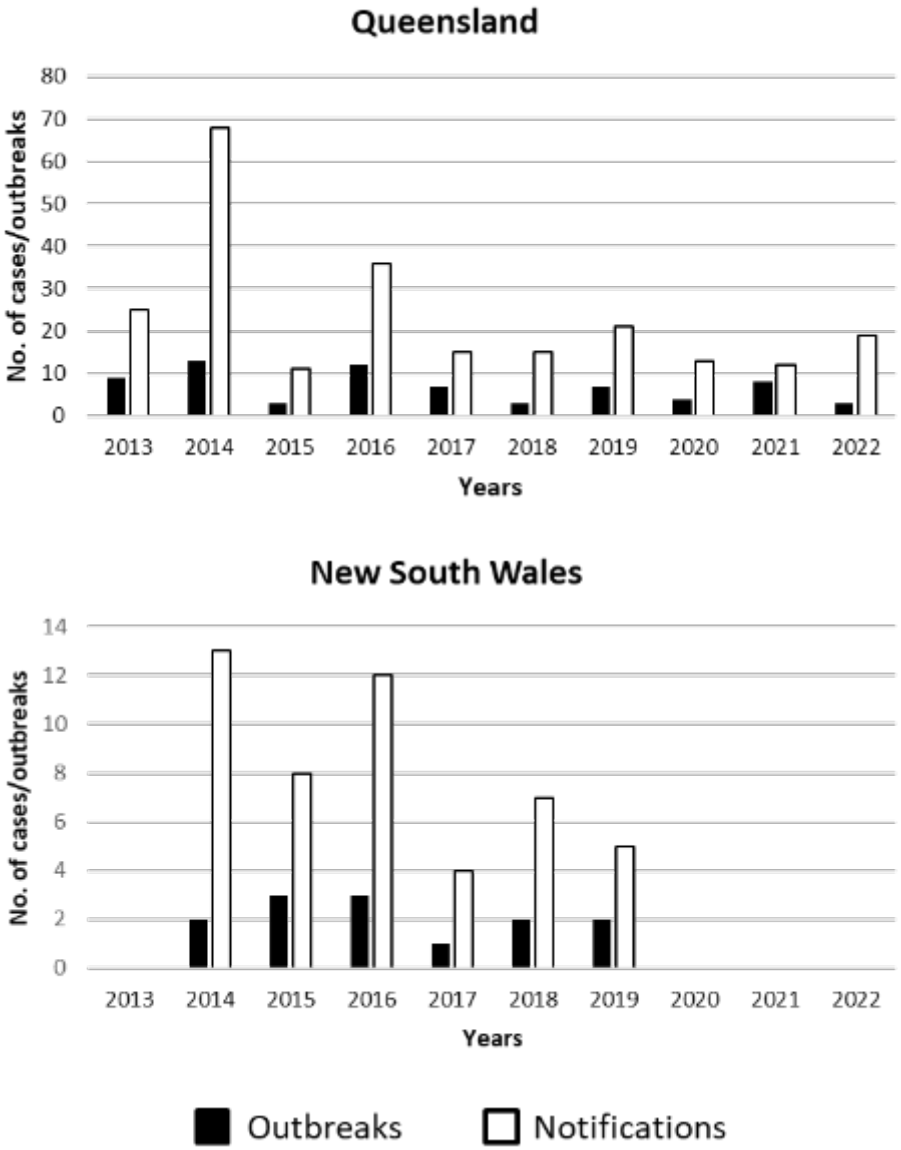


Figure 34. Ciguatera notifications and outbreaks, QLD and NSW, 2013 - 2022 (Farrell et al., 2016a, 2016b, Edwards et al., 2019, Szabo et al., 2022).

CTX remains a significant risk for the fishing industry and Australian seafood consumers (Table A6). The work conducted under this project has opened several lines of enquiry that show promise for future advancements, particularly with rapid test kits. Unfortunately, none of the analytical methods currently available are suitable for real-time risk management as they are expensive, require laborious extraction of toxins prior to analysis, and this can only be done in a laboratory setting.

13 Recommendations

13.1 Public health

- New evidence from this project does not support a change to current CP risk management for Spanish Mackerel in Australia.. Risk management should continue to include size restrictions and prohibitions on sale of fish caught in known CP 'hot spots'.
- Maintenance of education for consumers and fishers is important to promote awareness on the potential risks of CP. This education should cover the entire QLD and NSW coastline because of the high likelihood of Spanish Mackerel ranging further into southern NSW waters as sea temperatures increase and the EAC pushes further southwards.
- As CTXs have been found to be higher in liver and viscera than fillets, recommendations that Spanish Mackerel be gutted prior to sale may be considered.
- Consumer education should include advice on avoiding cooking and eating the head, roe, liver or other viscera as CTXs are concentrated in these parts and may increase exposure.
- Engage with health agencies to improve data collection on CP illnesses, involving GPs and health organisations would provide valuable data needed to improve risk assessment.
- Review current CP monitoring and response to ensure case data (food consumption, fish size, etc) is collected and samples submitted for CTX analysis where possible.
- Investigate support for development of a market for frozen product, which could lead to a 'test and release' approach. Results obtained in this process would lead to valuable data to better assess and manage this risk.
- Australian food safety management should take note of recommendations of the Codex Committee on Contaminants in Foods (CCCF16) 'Code of practice for the prevention or reduction of Ciguatera Poisoning' when they are released later in 2024.

13.2 Analytical

- Future research on CTX detection needs to focus on the sample extraction procedure, as it currently requires a well-equipped chemical laboratory, takes 6+ hours, and can show relatively low toxin recovery rates. A faster extraction protocol would enable all CTX detection methods: LC-MS, ELISA and cell bioassays to be conducted in a more timely and cost effective manner, as well as improving toxin recovery rates.
- The ELISA test kits showed considerable promise in detection of CTX, especially at low concentrations. However, they are not currently fit for purpose for use at point of sale or in the field as they require a chemical analysis laboratory in order to undertake sample extraction. Further research should address the challenges of baseline drift, validate the kit for use with P-CTX-1B in key fish species, and determine the LoD for this method.
- The CTX ELISA kit can be used as a pre-screening tool in future research as it is sensitive and more cost-effective than LC-MS. Other CTX detection including biosensors need to be considered in the scope of future detection approaches.

13.3 Environmental and Biological studies

- The approach taken here to include an understanding of fish biology and migration, as well as environmental parameters, has been useful to better understand the complex issue of CTX distribution along the Australian coastline. We recommend similar approaches in future work.

- Further fish sampling is recommended to better underpin risk management. Initially this should focus on known risk species and hot spots in order to increase the prevalence of CTX detection and therefore maximise information collected.

- While Spanish Mackerel is a known hazard, other fish species such as Coral Trout are leading causes of CP, particularly in QLD. The risk of CP may be simpler to mitigate in a fish with a more localised home range, rather than one that migrates long distances. Future research on other leading CP vectors is important.

- On-going fundamental research on Spanish Mackerel stocks using population genetic approaches in combination with CTX analyses would be useful in understanding risk in relation to population biological factors, migratory patterns and potential feeding areas where CTX uptake may occur.

- Further research analysing environmental correlates of CP and CTXs is needed to understand the proximate causes of changes in CP frequency. Internationally, climate change is expected to lead to increases in CP due to increasing cyclones, storms, coral damage and marine heatwaves. The impact of these factors in Australia is not known and needs to be investigated.

14 Appendices

Figures

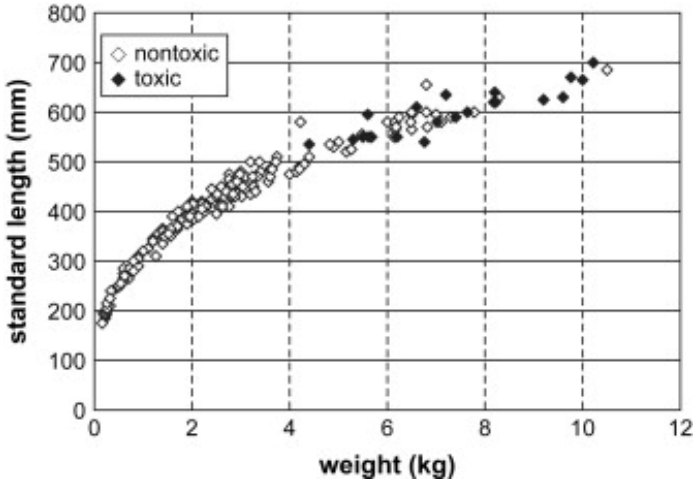


Figure A 1. Size of toxic specimens of *L. monostigma* (Onespot Snapper) (Oshiro et al., 2010)

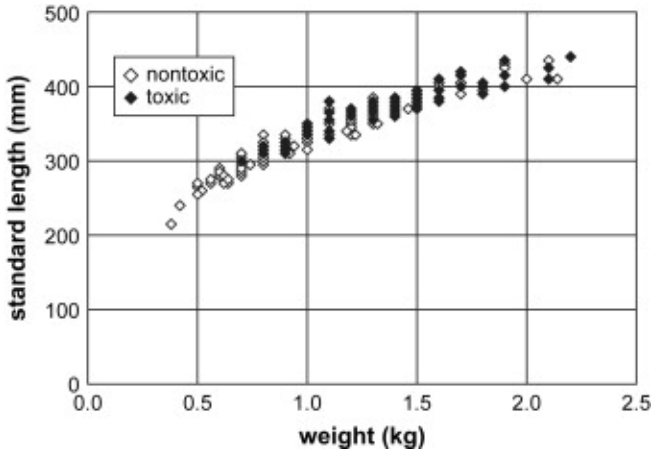


Figure A 2. Size of toxic specimens of *E. fuscoguttatus* (Flowery Rockcod, Oshiro et al., 2010)

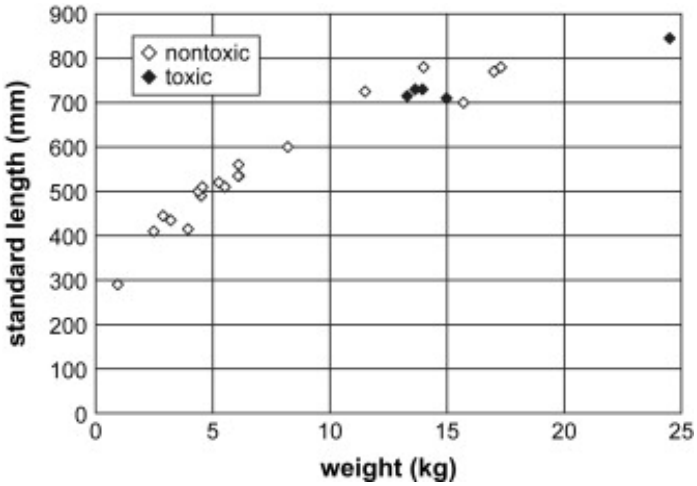


Figure A 3. Size dependency of toxic specimens of *L. bohar* (Red Bass, Oshiro et al., 2010).

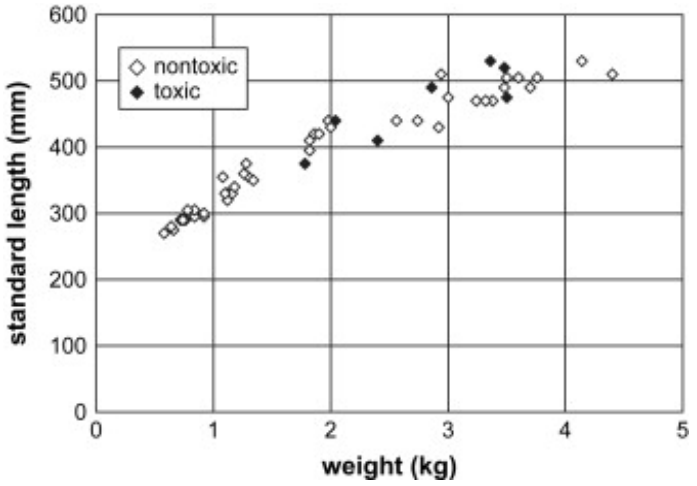


Figure A 4. Size dependency of toxic specimens of *V. louti* (Yellowedge Coronation Trout, Oshiro et al., 2010).

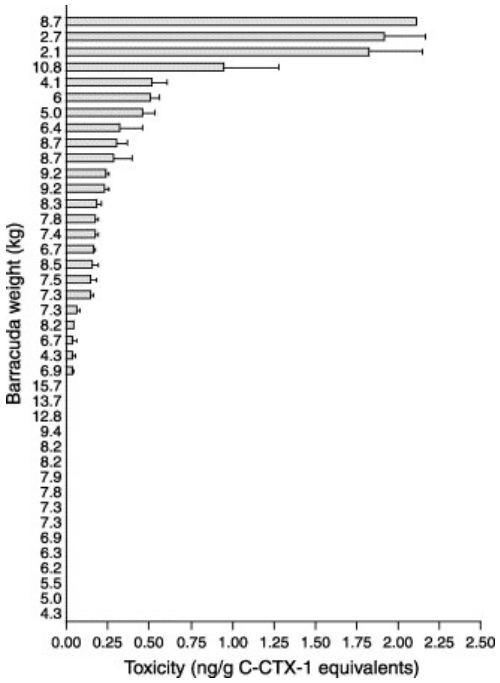
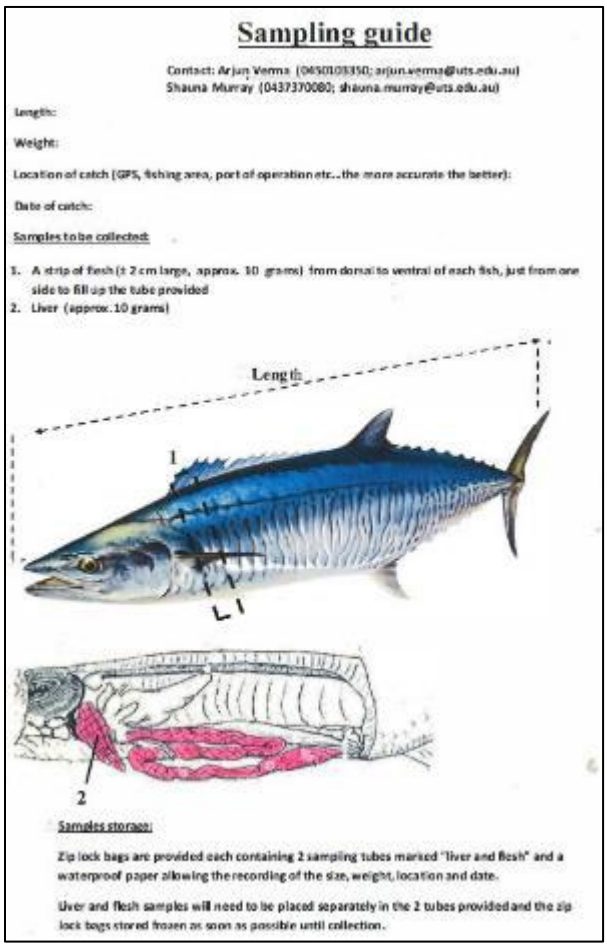


Figure A 5. Caribbean ciguatoxin C-CTX-1 equivalents measured in liver specimens of 40 *Sphyraena barracuda (*Barracuda) caught off the coast of Marathon Key, FL, USA by cytotoxicity assay. Each column, assigned with the weight of each fish, represents the mean±SEM (*n*=3 except for the fish weighing 8.7 kg) (Dechraoui et al., 2005).



Sampling guide

Contact: Arjun Verma (0450103350; arjun.verma@uts.edu.au)
Shauna Murray (0437370080; shauna.murray@uts.edu.au)

Length:

Weight:

Location of catch (GPS, fishing area, port of operation etc...the more accurate the better):

Date of catch:

Samples to be collected:

1. A strip of flesh (± 2 cm large, approx. 10 grams) from dorsal to ventral of each fish, just from one side to fill up the tube provided
2. Liver (approx. 10 grams)

Length

1

L I

2

Samples storage:

Zip lock bags are provided each containing 2 sampling tubes marked "liver and flesh" and a waterproof paper allowing the recording of the size, weight, location and date.

Liver and flesh samples will need to be placed separately in the 2 tubes provided and the zip lock bags stored frozen as soon as possible until collection.

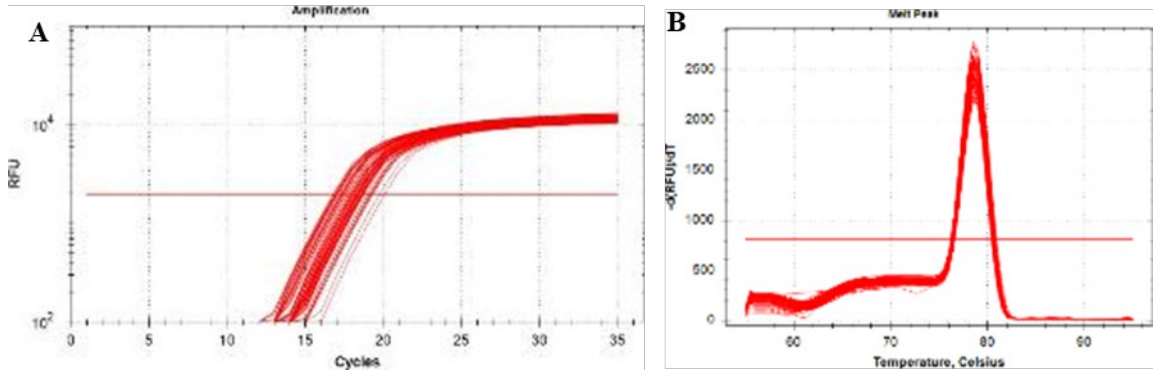


Figure A 7. qPCR amplification curve displaying Ct values and showing that the identity of all specimens was *S. commerson* ; B. Melt curve analysis, for fish specimens collected during 2021-22 fishing season.

Tables

Table A 1. Average CTX content in samples from 2021 fishing season.

2021 Season		
		µg/kg
n =	12	
Average (ng/mL)	0.44	0.09
SD	0.18	
RSD	42%	
Highest (ng/mL)	0.8	
Lowest (ng/mL)	0.2	

Table A 2. Average CTX content in samples from 2022 fishing season.

2022 Season		
		µg/kg
n =	8	
Average (ng/mL)	0.30	0.06
SD	0.14	
RSD	46%	
Highest (ng/mL)	0.532	
Lowest (ng/mL)	0.161	

Table A 3. Recovery values (%) of samples spiked with P-CTX-1B

Fish ID	**Recovery of P-CTX-1B (%)**

UTS 17F	22
UTS 17F #2	18
UTS 17F #3	19
UTS 114	17
UTS 146	16
UTS178	10
UTS 201	9
FRDC229F	18
MAC117F	16

Table A 4. The known congeners of CTXs and the source they were originally described from.

Origin	**Toxin Name**	**Molecular Ion [M +H]$^+$**	**Source**	**Toxicity[1]**
Pacific (type I)	CTX1B (Murata et al., 1990a), CTX-1(Lewis et al., 1991)	1111.6 (Murata et al., 1990a; Lewis et al., 1991)	Giant Moray (*Gymnothorax javanicus*) (Murata et al., 1990a) Giant Moray (*Gymnothorax javanicus*) (Lewis et al., 1991)	CTX1B- 0.35 µg/kg (Murata et al., 1990a) CTX-1- 0.25 µg/kg (Lewis et al., 1991)
	52-epi-54-deoxy-CTX-1 (CTX-2)	1095.5(Lewis et al., 1991)	Giant Moray (*Gymnothorax javanicus*) (Lewis et al., 1991)	2.3 µg/kg (Lewis et al., 1991)
	54-deoxy-CTX-1B (CTX-3)	1095.5(Lewis et al., 1991)	Giant Moray (*Gymnothorax javanicus*) (Lewis et al., 1991)	0.9 µg/kg (Lewis et al., 1991)
	CTX4A	1061.6 (Yasumoto et al., 2000)	*Gambierdiscus sp.* (Yasumoto et al., 2000) *G. polynesiensis* (Chinain et al., 2010)	12 µg/kg (Chinain et al., 2010)
	CTX4B	1061.6 (Yasumoto et al., 2000)	*Gambierdiscus sp.* (Yasumoto et al., 2000) *G. polynesiensis* (Chinain et al., 2010)	20 µg/kg (Chinain et al., 2010)

Pacific (Type II)	CTX3C	1023.6 (Satake et al., 1993)	*Gambierdiscus sp.* (Satake et al., 1993) *G. polynesiensis* (Chinain et al., 2010)	2.5 µg/kg (Chinain et al., 2010)
	49-epi-CTX-3C	1023.6 (Chinain et al., 2010)	*Gambierdiscus sp.* (Satake et al., 1993) *G. polynesiensis* (Chinain et al., 2010)	8 µg/kg (Chinain et al., 2010)
	M-seco-CTX- 3C	1041.6 (Chinain et al., 2010)	*Gambierdiscus sp.* (Satake et al., 1993) *G. polynesiensis* (Chinain et al., 2010)	10 µg/kg (Chinain et al., 2010)
Caribbean	C-CTX-1	1141.6 (Vernoux & Lewis, 1997; Pottier et al., 2002)	Horse-eye jack (*Caranx latus*)	3.6 µg/kg (Vernoux & Lewis, 1997)
	C-CTX-2	1141.6 (Vernoux & Lewis, 1997; Pottier et al., 2002)	Horse-eye jack (*Caranx latus*)	Toxic (Vernoux & Lewis, 1997)
Indian	I-CTX-1	1141.6 (Hamilton et al., 2002b)	Red Bass (*Lutjanus bohar*) Red Emperor (*Lutjanus sebae*)(Hamilton et al., 2002b)	Toxic (Hamilton et al., 2002b)

[1]LD_{50} doses calculated via i.p. injection in mice

Table A 5. CTXs detected in seafood in Australia and the method of detection.TLC: thin layer chromatography, DLBA: Diptera Larvae Bio Assay.

Latin name (Common name)	**Source**	**CTX**	**Method of detection**
Barracuda			
Sphyraena jello (Pickhandle Barracuda)	Hervey Bay, QLD, Australia (Lewis & Endean, 1984)	CTX – positive (Lewis & Endean, 1984)	TLC & MBA (Lewis & Endean, 1984)
Eel			
Gymnothorax javanicus (Giant Moray)	QLD, Australia (Lewis & Jones, 1997), (Lewis et al., 1991)	CTX-1, CTX-4B CTX-2 CTX-3 P-CTX-1 P-CTX-2 P-CTX-3 and analogues of CTX 3C: 2,3-dihydroxyCTX3C and 51-hydroxyCTX3C	HPLC/MS (Lewis & Jones, 1997; Satake et al., 1998), HPLC/HNMR (Legrand et al., 1989; Murata et al., 1990a; Lewis et al., 1991), TLC (Scheuer et al., 1967),

Latin name (Common name)	Source	CTX	Method of detection
		(Lewis & Jones, 1997), (Lewis et al., 1991; Satake et al., 1998)	DLBA (Labrousse & Matile, 1996), MBA (Scheuer et al., 1967; Lewis & Jones, 1997; Satake et al., 1998)
Grouper/Coral Trout			
Plectropomus spp. (Coral Trout)	Great Barrier Reef, Australia (Lewis & Sellin, 1992)	CTX-1 (Lewis & Sellin, 1992), CTX-2 (Lewis & Sellin, 1992), CTX-3 (Lewis & Sellin, 1992)	HPLC/MS (Lewis & Sellin, 1992), MBA (Lewis & Sellin, 1992)
Grunt			
Pomadasys maculatus (Blotched Javelin)	Platypus Bay, QLD, Australia (Lewis & Sellin, 1992)	CTX-1 (Lewis & Sellin, 1992), CTX-2 (Lewis & Sellin, 1992), CTX-3 (Lewis & Sellin, 1992)	HPLC/MS (Lewis & Sellin, 1992), MBA (Lewis & Sellin, 1992)
Mackerel			
Scomberomorus commerson (Spanish Mackerel)	Hervey Bay, QLD, Australia (Lewis & Endean, 1984), Hervey Bay, QLD, Australia (Endean et al., 1993)	CTX-1 (Lewis & Sellin, 1992), CTX-2 (Lewis & Sellin, 1992), CTX-3 (Lewis & Sellin, 1992)	HPLC/MS (Lewis & Sellin, 1992), TLC (Endean et al., 1993), MBA (Lewis & Endean, 1984; Lewis & Sellin, 1992; Endean et al., 1993)

Table A 6. Schedule of Ciguatera High Risk Areas provided by Sydney Fish Market (SFM, 2015).

Prohibited species – To be rejected	
Chinamanfish (*Symphorus nematophorus*) Tripletail Maori Wrasse (*Cheilinus trilobatus*) Humphead Maori Wrasse (*Cheilinus undulatus*) Red Bass (*Lutjanus bohar*) Paddletail (*Lutjanus gibbus*) Giant Moray (*Gymnothorax javanicus*)	
Prohibited supply regions- reject consignments of listed species caught in these regions	
Region	**Species**
Kiribati	All warm water ocean fish
The following Queensland waters: Platypus Bay on Fraser Island, bounded by the co-ordinates: GPS South 25 – 01 – 991; North 153 – 11 – 761	All warm water ocean fish Spanish Mackerel (*Scomberomrous commerson*) Mackerel (*Scomberomrous* spp.) – excluding Spotted and School Mackerel under 6 kg.
Marshall Islands	All warm water ocean fish
New Caledonia and Capel Bank	All warm water ocean fish

The following Northern Territory waters: Bremer Island Bonner Rocks Miles Island	The following species: Pickhandle Barracuda (*Sphyraena jello*) Bluespotted Rockcod (*Cephalopholis cyanostigmata*)

Immediate vicinity of Cape Arnhem North East Island and Connexion Island (both near Groote Eylandt Gove Peninsula, in the immediate vicinity of Nhulunbuy)	Coral Trout (*Plectropomus* spp. & *Variola* spp.) Red Emperor (*Lutjanus sebae*) Queensland Groper (*Epinephelus lanceolatus*) Trevally (*Caranx* spp.)
Fijian waters	Coral Trout (*Plectropomus* spp. & *Variola* spp.)

Table A 7. Maximum size limit for high risk species (SFM, 2015).

Species	Size Limit (Maximum whole size in Kg)				
	NSW	QLD	NT	WA	Pacific countries
Pickhandle Barracuda (*Sphyraena jello*)		10			10
Coral Rockcod (*Cephalopholis spp.* and *Cephalopholis miniata*)		3			3
Coral Trout (*Plectropomus spp*. and *Variola spp.)*	6	6	6	6	Reject
Yellowtail Kingfish & Samsonfish (*Seriola spp*.)		10			10
Mackerel (various), except Spanish Mackerel (*Scomberomorus spp*.)	10	10			10
Giant Queenfish (*Scomberoides commersonianus*)		10			10
Red Emperor (*Lutjanus sebae*)		6			6
Reef Cods Goldspotted Rockcod (*Epinephelus coioides*) Flowery Rockcod (*Epinephelus fuscoguttatus*) Queensland Groper (*Epinephelus lanciolatus*) Greasy Rockcod (*Epinephelus tauvina*)		10			10
Surgeonfish (All *Acanthuridae* family members)		10			Reject
Spangled Emperor (*Lethrinus nebulosus*)		6			6
Spanish Mackerel (*Scomberomorus commerson*)	10 *	8 *			10
Trevally (*Caranx spp.*)		6			6
Tuskfish (*Choerodon spp*.)		6			6

* 10 kg whole or 8 kg gutted & headed

Table A 8. LC-MS analysis of P-CTX-1B in samples of *S. commerson* flesh and liver collected in 2015, and from an analysis of fish implicated in CP events in NSW in 2014 (at end of Table).

Sample Code	Location	Date of Catch	Length (cm)	Weight (kg)	P -CTX-1B in flesh (µg kg^{-1})[1]	P-CTX-1B in liver (µg kg^{-1})[1]
AIMS-1	Davies Reef, QLD	2/01/15	149	21	ND	ND
AIMS-2	Davies Reef, QLD	2/01/15	105	6	ND	ND
AIMS-4	Port Douglas, QLD (14°.47.88S 149°.25.18E)	12/01/15	134	13.5	<0.1	<0.4
AIMS-5	Port Douglas, QLD (14°.47.88 S 149°.25.18 E)	--	136	16	0.13	1.39
AIMS-6	Great Barrier Reef, Rockhampton, QLD (22°.00.48 S 152°.38.85 E)	23/01/15	110	6.3	<0.1	ND
AIMS-10	Whitsundays, QLD (Reef No: 19-138)	12/01/15	106	6.1	<0.1	<0.4
AIMS-11	Whitsundays, QLD (Reef No: 19-138)	13/01/15	120	11.9	<0.1	<0.4

AIMS-12	Townsville, QLD (19°.47.88S 144°.25.18E)	12/01/15	117	11.2	<0.1	<0.4
AIMS-13	Whitsundays, QLD (20°.01.45S-149°.41.02E)	13/01/15	103	5.8	ND	ND
SFM-3	Brunswick Heads, NSW	2/02/15	120	8	ND	ND
SFM-16	Mooloolaba, QLD	6/01/15	96	6	ND	ND
SFM-19	Port Bundaberg, QLD	18/12/14	120	9.4	ND	ND
SFM-33	Mooloolaba, QLD	14/01/15	149	24	ND	ND
SFM-34	Mooloolaba, QLD	16/01/15	133	17	ND	ND
CF-B-1	Coffs harbour, NSW	12/02/15	110	12	ND	ND
CF-B-2	Split island, Coffs Harbour, NSW	19/02/15	125	12.2	ND	ND
CF-B-8	Lighthouse, Coffs Harbour, NSW	10/02/15	130	13.6	ND	ND
CF-B-16	Patch, Coffs Harbour, NSW	2/03/15	131	13.3	ND	ND
CF-B-19	Patch, Coffs Harbour, NSW	2/03/15	130	12.5	ND	ND
CF-B-22	Lighthouse, Coffs Harbour, NSW	12/02/15	120	11.1	ND	ND

CF-B-25	Coffs Harbour, NSW	23/01/15	110	12	ND	ND
CF-B-26	South Solitary island, Coffs Harbour, NSW	26/02/15	128	15.8	ND	ND
CF-B-27	Patch, Coffs Harbour, NSW	2/03/15	124	11.2	ND	ND
CF-B-28	South Solitary island, Coffs Harbour, NSW	26/02/15	143	20.5	ND	ND
CF-B-30	Patch , Coffs Harbour, NSW	28/02/15	125	11.2	ND	ND
CF-D-3	Evans Head, NSW	5/03/15	150	23.6	ND	ND
CF-C-2	Evans Head, NSW	28/04/15	129	13.5	ND	ND
CF-C-5	Black Head, NSW	26/03/15	129	13.1	ND	ND
CF-C-10	Evans Head, NSW	28/04/15	127	12.5	ND	ND
CF-C-11	Ballina, NSW	12/03/15	128	11.2	ND	<0.4
CF-C-13	Evans Head, NSW	28/04/15	124	12.5	ND	ND
CF-C-22	Ballina, NSW	12/03/15	142	19.5	ND	<0.4
CF-E-5	Brunswick Head, NSW	26/03/15	110	10.5	ND	ND
CF-E-12	Brunswick Head, NSW	21/03/15	120	13	ND	ND
CF-E-16	Brunswick Head, NSW	9/04/15	110	11	ND	ND

CF-E-21	Brunswick Head, NSW	27/03/15	120	12	ND	ND
CF-E-22	Brunswick Head, NSW	5/04/15	90	9	ND	ND
CF-E-24	Brunswick Head, NSW	21/01/15	90	9	ND	ND
CF-E-27	Brunswick Head, NSW	14/02/15	100	10	ND	ND
CF-E-28	Brunswick Head, NSW	26/01/15	95	9	ND	ND
CF-E-30	Brunswick Head, NSW	29/03/15	110	8	ND	ND
RF-Q-2	Byron Bay, NSW	19/04/15	80	4.5	ND	ND
RF-X-5	Byron Bay, NSW	19/04/15	90	6	ND	ND
RF-X-6	Byron Bay, NSW	4/03/15	120	12	ND	ND
RF-T-1	Byron Bay, NSW	4/03/15	95	7	ND	ND
RF-F-1	Coffs Harbour, NSW	18/04/15	124	15	ND	ND
RF-H-1	Coffs Harbour, NSW	20/03/15	95	10	ND	ND
RF-H-2	Coffs Harbour, NSW	20/03/15	98.5	7	ND	<0.4
RF-H-3	Coffs Harbour, NSW	20/03/15	100	12	ND	ND
RF-H-4	Coffs Harbour, NSW	23/03/15	95	9	ND	ND
RF-H-5	Coffs Harbour, NSW	26/03/15	90	8	ND	ND
RF-H-6	Coffs Harbour, NSW	26/03/15	100	12	ND	ND
RF-J-1	Solitary island, Coffs Harbour, NSW	2/04/15	135	12	ND	ND

RF-J-2	Coffs Harbour, NSW	23/04/15	110	11.5	ND	ND
RF-J-3	Split Solitary, Coffs Harbour, NSW	19/04/15	145	17.5	ND	ND
RF-M-1	Coffs Harbour, NSW (30°.17S 153°. 10E)	15/03/15	110	11	ND	<0.4
RF-M-2	Coffs Harbour, NSW (30°.22S 153°. 50E)	31/03/15	120	12	ND	ND
RF-M-3	Coffs Harbour, NSW (30°.75S 153°. 10E)	15/03/15	115	11.5	ND	ND
RF-M-4	Coffs Harbour, NSW (30°.22S 153°. 50E)	31/03/15	130	19	ND	ND
RF-M-5	Macqualies, Coffs Harbour, NSW	1/04/15	120	14.5	ND	ND
RF-M-6	Coffs Harbour, NSW	2/04/15	129	18.7	ND	ND
RF-N-1	Coffs Harbour, NSW	7/03/15	123	11	ND	ND
RF-N-2	Coffs Harbour, NSW	29/03/15	140	14.7	ND	ND
RF-N-3	Coffs Harbour, NSW	26/04/15	120	17	ND	ND
RF-N-4	Coffs Harbour, NSW	30/05/15	110	11	ND	ND
RF-Y-1	Coffs Harbour, NSW	5/04/15	118	14.8	ND	ND
RF-Y-2	Coffs Harbour, NSW	5/04/15	127	19.8	ND	ND
RF-Y-3	Coffs Harbour, NSW	5/04/15	134	19.2	ND	ND

RF-Y-4	Coffs Harbour, NSW	19/04/15	131.5	16.2	ND	ND
RF-Y-5	Coffs Harbour, NSW	7/04/15	135	19.4	ND	ND
RF-Z-1	Coffs Harbour, NSW	3/04/15	132	18.9	ND	ND
RF-Z-2	Coffs Harbour, NSW	3/04/15	134.5	19	ND	ND
RF-Z-3	Coffs Harbour, NSW	3/04/15	117	14.2	ND	ND
RF-Z-4	Coffs Harbour, NSW	3/04/15	135	19.4	ND	ND
RF-Z-5	Coffs Harbour, NSW	4/04/15	120	14.5	ND	ND
RF-AA-1	Coffs Harbour, NSW	6/04/15	130.4	16	ND	ND
RF-AA-2	Coffs Harbour, NSW	10/04/15	117	14	ND	ND
RF-AA-3	Coffs Harbour, NSW	14/04/15	134.5	19.2	ND	ND
RF-AA-5	Coffs Harbour, NSW	12/04/15	133	18.9	ND	ND
RF-AP-1	South Solitary island, Coffs Harbour, NSW	30/05/15	142	16	<0.1	<0.4
RF-AP-2	North Solitary island, Coffs Harbour, NSW	30/05/15	145	17	ND	ND
RF-AB-1	Forster, NSW	6/04/15	125	13	ND	ND
RF-AC-1	Forster, NSW	6/04/15	120	12	ND	ND
RF-AD-1	Coffs Harbour, NSW	31/03/15	134	14.6	ND	ND
V1207-A	Scott’s Head, NSW2	2/3/14	--	25.7	0.4	NT

V1207-B	Evans Head, NSW2	13/2/14	--	10	0.6	NT
V1207-C3	Evans Head, NSW2	13/2/14	--	17	1.0	NT
V1207-D4	Evans Head, NSW2	13/2/14	--	3.40	ND	NT

ND: Not detected; NT: Not tested

[1]LC-MS analysis was performed at the Cawthron Institute, Nelson, New Zealand

[2]Results related to CFP in NSW in 2014, obtained from the NSW Food Authority (Farrell *et al.*, 2016)

[3]Three flesh fillets were tested from 2 specimens of Spanish Mackerel from Evans Head in 2014, which were 10 and 17 kg. Unfortunately, the NSW Food Authority was not able to verify exactly which of the three fillets came from which fish.

Table A 9. LC–MS/MS and ELISA analyses of P-CTX-1B in samples of *S. commerson* flesh and liver collected during 2021-22 fishing season.

na: data not available; * refers to values determined from equations as stated in Mackie et al. (2003); <LOD: below the limit of detection; <LOQ: below the limit of quantification.

S. no.	Sample code	Date of collection	Tail length (in mm)	fork length (in mm)	Weight (in Kgs)	Location	P-CTX-1B in flesh (µg/kg)	P-CTX-1B in liver (µg/kg)	P-CTX-1B in flesh ELISA (µg/kg)	P-CTX-1B in liver ELISA (µg/kg)
1	FRDC 1	8/12/2021	1080.0	980.0	7.3*	Fraser Inshore	<LOD	<LOD	<LOQ	
2	FRDC 2	8/12/2021	1039*	940.0	6.4*	Fraser Inshore	<LOD	<LOD		
3	FRDC 3	8/12/2021	970.0	860.0	4.9*	Fraser Inshore	<LOD	<LOD		<LOQ
4	FRDC 4	8/12/2021	960.0	850.0	4.7*	Fraser Inshore	<LOD	<LOD	<LOQ	
5	FRDC 5	8/12/2021	1080.0	970.0	7.1*	Fraser Inshore	<LOD	<LOD		

6	FRDC 6	8/12/2021	1200.0	1080.0	9.9 *	Fraser Inshore	<LOD	<LOD		
7	FRDC 7	8/12/2021	990.0	910.0	5.8*	Fraser Inshore	<LOD	<LOD		
8	FRDC 8	8/12/2021	1000.0	920.0	6.0*	Fraser Inshore	<LOD	<LOD		
9	FRDC 9	8/12/2021	950.0	850.0	4.7*	Fraser Inshore	<LOD	<LOD		
10	FRDC 10	8/12/2021	980.0	860.0	4.9*	Fraser Inshore	<LOD	<LOD		
11	FRDC 12	8/12/2021	1010.0	910.0	5.8*	Fraser inshore	<LOD	<LOD	<LOQ	
12	FRDC 13	8/12/2021	912*	820.0	4.2*	Fraser inshore	<LOD	<LOD	<LOQ	
13	FRDC 14	27/08/2021	1410.0	1330.0	19.0	Fraser inshore	<LOD	<LOD		
14	FRDC 15	14/11/2021	1007*	910.0	5.8*	Rockhampton offshore	<LOD	<LOD		
15	FRDC 16	27/08/2021	1240.0	1140.0	11.7*	Fraser Inshore	<LOD	<LOD		<LOQ
16	FRDC 17	7/12/2021	1040.0	920.0	6.0*	Fraser Inshore	<LOD	<LOD		
17	FRDC 18	7/12/2021	1010.0	870.0	5.0*	Fraser Inshore	<LOD	<LOD		<LOQ
18	FRDC 19	7/12/2021	990.0	910.0	5.8*	Fraser Inshore	<LOD	<LOD	0.005	
19	FRDC 20	9/12/2021	1040.0	940.0	6.4*	Fraser Inshore	<LOD	<LOD	<LOQ	<LOQ
20	FRDC 21	9/12/2021	810.0	710.0	2.7*	Fraser Inshore	<LOD	<LOD	<LOQ	<LOQ
21	FRDC 22	9/12/2021	950.0	850.0	4.7*	Fraser Inshore	<LOD	<LOD		
22	FRDC 23	9/12/2021	950.0	860.0	4.9*	Fraser Inshore	<LOD	<LOD		
23	FRDC 24	9/12/2021	933*	840.0	4.5*	Fraser Inshore	<LOD	<LOD		
24	FRDC 26	9/12/2021	950.0	840.0	4.5*	Fraser Inshore	<LOD	<LOD		
25	FRDC 27	9/12/2021	950.0	840.0	4.5*	Fraser Inshore	<LOD	<LOD		
26	FRDC 29	9/12/2021	950.0	855.9*	4.8*	Fraser Inshore	<LOD	<LOD		
27	FRDC 30	16/11/2021	1100.0	1000.0	7.8*	Fraser Inshore	<LOD	<LOD		
28	FRDC 31	16/11/2021	1367*	1249.3	15.6*	Fraser Inshore	<LOD	<LOD		
29	FRDC 32	16/11/2021	950.0	850.0	4.7*	Fraser Inshore	<LOD	<LOD		
30	FRDC 33	16/11/2021	950.0	840.0	4.5*	Fraser Inshore	<LOD	<LOD		
31	FRDC 34	16/11/202	1010.0	910.0	5.8*	Fraser Inshore	<LOD	<LOD		

32	FRDC 35	16/11/2021	990.0	900.0	5.6*	Fraser Inshore	<LOD	<LOD	<LOQ

33	FRDC 39	26/09/2021	1293.5*	1180.0	13.1*	Fraser Inshore	<LOD	<LOD		
34	FRDC 40	26/09/2021	1510.0	1390.0	21.8*	Fraser Inshore	<LOD	<LOD	0.010	0.010
35	FRDC 41	26/08/2021	1208.7*	1100.0	10.5*	Fraser Inshore	<LOD	<LOD	0.006	
36	FRDC 43	26/08/2021	1460.0	1320.0	18.5*	Fraser Inshore	<LOD	<LOD		
37	FRDC 44	26/08/2021	1360.0	1290.0	17.2*	Fraser Inshore	<LOD	<LOD	0.008	<LOQ
38	FRDC 45	26/08/2021	1198*	1090.0	10.2*	Fraser Inshore	<LOD	<LOD	<LOQ	<LOQ
39	FRDC 48	20/12/2021	1145*	1040.0	8.8*	Fraser Inshore	<LOD	<LOD	0.010	<LOQ
40	FRDC 58	25/03/2022	1220.0	1110.6*	11.0	Teewah	<LOD	<LOD		
41	FRDC 59	1/02/2022	1410.0	1289.9*	17.0	Sunshine reef	<LOD	<LOD		
42	FRDC 77	24/12/2021	980.0	884.2*	5.3*	Jew Shoal, Laguna Bay	<LOD	<LOD		
43	FRDC 78	15/01/2022	1210.0	1101.2*	11.0	Laguna bay, Noosa	<LOD	<LOD		
44	FRDC 79	26/01/2022	990.0	893.6*	5.6	Sunshine reef (off Noosa heads)	<LOD	<LOD		
45	FRDC 80	26/01/2022	1010.0	912.5*	6.1	Sunshine reef (off Noosa heads)	<LOD	<LOD		
46	FRDC 81	25/03/2022	1310.0	1195.5*	20.0	Fraser Waddy	<LOD	<LOD		
47	FRDC 82	25/03/2022	1050.0	950.2*	8.0	Fraser Waddy Point	<LOD	<LOD		
48	FRDC 83	25/03/2022	1130.0	1025.7*	8.0	Fraser Waddy	<LOD	<LOD		
49	FRDC 91	23/03/2022	1230.0	1120.1*	10.0	Fraser Waddy	<LOD	<LOD		<LOQ
50	FRDC 92	23/03/2022	1100.0	997.4*	7.5	Fraser Waddy	<LOD	<LOD	<LOQ	<LOQ
51	FRDC 93	23/03/2022	1210.0	1101.2*	9.5	Fraser Waddy	<LOD	<LOD		
52	FRDC 94	21/03/2022	1330.0	1214.4*	14.5	Fraser Waddy Point	<LOD	<LOD		
53	FRDC 101	17/01/2022	1020.0	920.0	6.0*	Fraser Inshore	<LOD	<LOD		
54	FRDC 102	17/01/2022	1050.0	940.0	6.4*	Fraser Inshore	<LOD	<LOD		
55	FRDC 103	17/01/2022	1060.0	970.0	7.1*	Fraser Inshore	<LOD	<LOD		

56	FRDC 104	17/01/2022	1040.0	960.0	6.9*	Fraser Inshore	<LOD	<LOD
57	FRDC 105	17/01/2022	1060.0	960.0	6.9*	Fraser Inshore	<LOD	<LOD
58	FRDC 106	17/01/2022	1100.0	1010.0	8.0*	Fraser Inshore	<LOD	<LOD

59	FRDC 107	17/01/2022	1160.0	1040.0	8.8*	Fraser Inshore	<LOD	<LOD		
60	FRDC 109	17/01/2022	1270.0	1180.0	13.1*	Fraser Inshore	<LOD	<LOD	0.006	<LOQ
61	FRDC 110	17/01/2022	970.0	870.0	5.0*	Fraser Inshore	<LOD	<LOD		
62	FRDC 112	17/01/2022	1160.0	1080.0	9.9*	Fraser Inshore	<LOD	<LOD	0.012	0.009
63	FRDC 113	17/01/2022	980.0	870.0	5.0*	Fraser Inshore	<LOD	<LOD		
64	FRDC 114	25/01/2022	960.0	850.0	4.7*	Hervey bay	<LOD	<LOD		
65	FRDC 115	25/01/2022	770.0	660.0	2.1*	Hervey bay	<LOD	<LOD		
66	FRDC 116	25/01/2022	930.0	830.0	4.4*	Hervey bay	<LOD	<LOD		
67	FRDC 117	25/01/2022	1170.0	1050.0	9.1*	Hervey bay	<LOD	<LOD		
68	FRDC 118	25/01/2022	1000.0	920.0	6.0*	Hervey bay	<LOD	<LOD		
69	FRDC 119	25/01/2022	1040.0	910.0	5.8*	Hervey bay	<LOD	<LOD		<LOQ
70	FRDC 120	25/01/2022	970.0	860.0	4.9*	Hervey bay	<LOD	<LOD		
71	FRDC 121	25/01/2021	980.0	900.0	5.6*	Hervey bay	<LOD	<LOD	<LOQ	
72	FRDC 122	25/01/2022	996.7*	900.0	5.6*	Hervey bay	<LOD	<LOD		
73	FRDC 123	25/01/2022	1110.0	970.0	7.1*	Hervey bay	<LOD	<LOD		
74	FRDC 124	25/01/2022	990.0	870.0	5.0*	Hervey bay	<LOD	<LOD		
75	FRDC 125	25/01/2022	1010.0	880.0	5.2*	Hervey bay	<LOD	<LOD	0.005	0.006
76	FRDC 126	26/01/2022	1250.0	1120.0	11.1*	Rockhampton offshore	<LOD	<LOD		
77	FRDC 127	26/01/2022	1120.0	990.0	7.5*	Rockhampton offshore	<LOD	<LOD		
78	FRDC 128	26/01/2022	1030.0	910.0	5.8*	Rockhampton offshore	<LOD	<LOD		
79	FRDC 130	26/01/2022	1010.0	880.0	5.2*	Rockhampton offshore	<LOD	<LOD		
80	FRDC 131	26/01/2022	1070.0	970.0	7.1*	Rockhampton offshore	<LOD	<LOD		
81	FRDC 132	26/01/2022	1130.0	1000.0	7.8*	Rockhampton offshore	<LOD	<LOD		
82	FRDC 133	26/01/2022	970.0	870.0	5.0*	Rockhampton offshore	<LOD	<LOD	<LOQ	

83	FRDC 134	26/01/2022	1030.0	910.0	5.8*	Rockhampton offshore	<LOD	<LOD		<LOQ
84	FRDC 135	26/01/2022	975.5*	880.0	5.2*	Rockhampton offshore	<LOD	<LOD		
85	FRDC 136	26/01/2022	1120.0	990.0	7.5*	Rockhampton offshore	<LOD	<LOD	<LOQ	
86	FRDC 137	26/01/2022	1070.0	930.0	6.2*	Rockhampton offshore	<LOD	<LOD		
87	FRDC 138	26/01/2022	1200.0	1060.0	9.3*	Rockhampton offshore	<LOD	<LOD	<LOQ	
88	FRDC 139	24/01/2022	830.0	740.0	3.0*	Rockhampton offshore	<LOD	<LOD	<LOQ	
89	FRDC 140	24/01/2022	996.7*	900.0	5.6*	Rockhampton offshore	<LOD	<LOD		
90	FRDC 141	24/01/2022	1090.0	990.0	7.5*	Rockhampton offshore	<LOD	<LOD		
91	FRDC 143	24/01/2022	1060.0	960.0	6.9*	Rockhampton offshore	<LOD	<LOD		
92	FRDC 144	24/01/2022	990.0	920.0	6.0*	Rockhampton offshore	<LOD	<LOD		
93	FRDC 145	24/01/2022	1050.0	960.0	6.9*	Rockhampton offshore	<LOD	<LOD		
94	FRDC 146	24/01/2022	1018*	920.0	6.0*	Rockhampton offshore	<LOD	<LOD		
95	FRDC 147	24/01/2022	1070.0	970.0	7.1*	Rockhampton offshore	<LOD	<LOD		
96	FRDC 148	24/01/2022	1071*	970.0	7.1*	Rockhampton offshore	<LOD	<LOD	<LOQ	
97	FRDC 149	1/02/2022	1081.5*	980.0	7.3*	Rockhampton offshore	<LOD	<LOD	<LOQ	
98	FRDC 150	1/02/2022	1124*	1020.0	8.3*	Rockhampton offshore	<LOD	<LOD		
99	REC 107	11/12/2021	1200.0	1091.8*	9.5	Sunshine Reef	<LOD	<LOD		
100	REC 108	16/12/2021	1550.0	1421.9*	26.0	Sunshine Reef	<LOD	<LOD		
101	REC 109	11/12/2021	1150.0	1044.6*	8.0	Sunshine reef	<LOD	<LOD		

102	REC 110	19/11/2021	1200.0	1091.8*	11.0	Coolum	<LOD	<LOD	<LOQ
103	REC 111	25/03/2022	1050.0	950.2*	7.0	Double Island	<LOD	<LOD	
104	REC 113	21/02/2022	1570.0	1440.8*	25.5	Sunshine reef	<LOD	<LOD	
105	REC 115	25/03/2022	1260.0	1148.4*	15.0	Double Island	<LOD	<LOD	
106	REC 145	8/06/2021	1300.0	1186.1*	13.3*	Coolum	<LOD	<LOD	
107	REC 148	27/11/2021	1000.0	903.1*	5.7*	Jew Shoal, Laguna Bay, Noosa	<LOD	<LOD	
108	FRDC 162	18/03/2022	1126.0	1021.9*	8.3*	Coffs harbour	<LOD	<LOD	
109	FRDC 161	18/03/2022	1358.0	1240.8*	15.3*	Coffs harbour	<LOD	<LOD	
110	FRDC 164	18/03/2022	1368.0	1250.2*	15.6*	Coffs harbour	<LOD	<LOD	
111	FRDC 173	25/03/2022	1075.0	938.0	6.4*	Wooli	<LOD	<LOD	
112	FRDC 184	7/05/2022	1013.0	948.0	6.6*	South West Rocks, Grassy Head	<LOD	<LOD	
113	FRDC 175	25/03/2022	1179.0	1038.0	8.8*	Wooli	<LOD	<LOD	
114	FRDC 182	25/03/2022	1233.0	1098.0	10.4*	Wooli	<LOD	<LOD	
115	FRDC 168	25/01/2022	1119.0	999.0	7.8*	The Wash, South Solitary	<LOD	<LOD	
116	FRDC 181	25/03/2022	1089.0	960.0	6.9*	Wooli	<LOD	<LOD	
117	FRDC 172	25/03/2022	1620.0	1488.0*	29.6	Wooli	<LOD	<LOD	
118	FRDC 174	25/03/2022	1042.0	917.0	5.9*	Wooli	<LOD	<LOD	
119	FRDC 171	25/03/2022	1084.0	955.0	6.7*	Wooli	<LOD	<LOD	
120	FRDC 186	3/02/2022	1461.0	1338*	19.3*	North Solitary Island	<LOD	<LOD	
121	RF AT 5	10/04/2022	na	na	10.2	Coffs harbour	<LOD	<LOD	
122	RF AS 6	16/04/2022	na	na	8.4	Coffs harbour	<LOD	<LOD	
123	RF AS 3	20/04/2022	na	na	8.0	Coffs harbour	<LOD	<LOD	
124	RF AS 4	15/04/2022	na	na	12.0	Coffs harbour	<LOD	<LOD	
125	RF AS 5	15/04/2022	na	na	8.0	Coffs harbour	<LOD	<LOD	
126	RF AT 3	10/04/2022	na	na	9.3	Coffs harbour	<LOD	<LOD	
127	RF AT 6	27/04/2022	na	na	8.6	Coffs harbour	<LOD	<LOD	
128	CH 7	28/04/2022	na	na	9.7	Coffs harbour	<LOD	<LOD	

129	CH 17	30/04/2022	na	na	11.5	Coffs harbour	<LOD	<LOD		
130	CH 13	27/04/2022	na	na	9.0	Coffs harbour	<LOD	<LOD		
131	CH 20	30/04/2022	na	na	8.0	Coffs harbour	<LOD	<LOD		
132	CH 24	27/04/2022	na	na	11.0	Coffs harbour	<LOD	<LOD		
133	CH 9	29/04/2022	na	na	12.0	Coffs harbour	<LOD	<LOD		
134	CH 12	28/04/2022	na	na	6.9	Coffs harbour	<LOD	<LOD		
135	CH 2	30/04/2022	na	na	7.7	Coffs harbour	<LOD	<LOD		
136	CH 30	29/04/2022	na	na	7.8	Coffs harbour	<LOD	<LOD		
137	CH 8	28/04/2022	na	na	11.5	Coffs harbour	<LOD	<LOD		
138	CH 11	28/04/2022	na	na	8.4	Coffs harbour	<LOD	<LOD		
139	CH 15	28/04/2022	na	na	8.3	Coffs harbour	<LOD	<LOD		
140	MAC 126	16/08/2022	1280.0	1167*	14.5	Wigton islands	<LOD	<LOD	0.007	0.012
141	FRDC 227	20/04/2022	1150.0	1010.0	8.0*	Rockhampton offshore	<LOD	<LOD		
142	FRDC 251	18/07/2022	1310.0	1200.0	13.8*	Fraser inshore	<LOD	<LOD		
143	MAC 117	7/08/2022	1200.0	1091.8*	10.4	Northern overfalls	<LOD	<LOD	<LOQ	
144	FRDC 226	20/04/2022	1090.0	950.0	6.6*	Rockhampton offshore	<LOD	<LOD	0.005	0.012
145	FRDC 229	20/04/2022	1060.0	930.0	6.2*	Rockhampton offshore	<LOD	<LOD	<LOQ	0.005
146	REC 434	6/05/2022	900.0	808*	4.3	Shipping channel	<LOD	<LOD		
147	REC 144	21/02/2022	1150.0	1044.6*	10.0	Maroola beach	<LOD	<LOD		
148	FRDC 221	20/04/2022	1080.0	960.0	6.9*	Rockhampton offshore	<LOD	<LOD		

Table A 10. LC–MS/MS and ELISA analyses of P-CTX-1B in samples of *S. commerson* flesh and liver collected during 2020-21 fishing season.

na: data not available; * refers to values determined from equations as stated in Mackie et al. (2003); <LOD: below the limit of detection; <LOQ: below the limit of quantification.

S. no.	Sample code	Date of collection	Tail length (in mm)	Fork length (in mm)	Weight (in Kgs)	Location	P-CTX-1B in flesh (µg/kg)	P-CTX-1B in liver (µg/kg)
1	BB bag 5	10/03/2021	1000	903	*na*	Byron	<LOD	<LOD
2	BB bag 3	10/03/2021	1300	1186	*na*	Byron	<LOD	<LOD
3	RF box AQ bag 3	16/02/2021	1050	950	*na*	Brunswick heads	<LOD	<LOD
4	Byron 95	12/02/2021	950	856	*na*	Ballina	<LOD	<LOD
5	Byron 124	12/02/2021	1240	1129	*na*	Ballina	<LOD	<LOD
6	CH bag 1	4/05/2021	1290	1177	16.5	Coffs harbour	<LOD	<LOD
7	RF box AR bag 4	29/04/2021	1300	1186	10	Coffs harbour	<LOD	<LOD
8	CH bag 5	4/05/2021	1250	1139	15.5	Coffs harbour	<LOD	<LOD
9	RF box AR bag 2	29/04/2021	1100	997	7.5	Coffs harbour	<LOD	<LOD
10	RF box AR bag 5	29/04/2021	1100	997	8	Coffs harbour	<LOD	<LOD
11	CH bag 4	4/05/2021	1150	1045	12.5	Coffs harbour	<LOD	<LOD
12	CH bag 21	13/05/2021	1440	1318	15.5	Coffs harbour	<LOD	<LOD
13	REC bag 356	15/05/2021	1560	1431	*na*	Coffs harbour	<LOD	<LOD
14	Fish 1	19/11/2020	1060	960	7	Bustard head	<LOD	<LOD
15	Fish 2	19/11/2020	1310	1196	15	Bustard head	<LOD	<LOD
16	Fish 3	19/11/2020	1510	1384	21.5	Bustard head	<LOD	<LOD
17	Fish 4	19/11/2020	980	884	6.6	Bustard head	<LOD	<LOD
18	AG1	29/04/2021	850	762	3.55	Coffs harbour	<LOD	<LOD
19	AG2	29/04/2021	1120	1016	8.95	Coffs harbour	<LOD	<LOD
20	AG3	28/02/2021	1130	1026	8	Fingal Island	<LOD	<LOD
21	AG4	29/04/2021	1300	1186	18.25	Coffs harbour	<LOD	<LOD
22	RF 31	11/02/2021	1100	997	7.1	Arrawarra	<LOD	<LOD
23	RF 32	12/02/2021	1230	1120	13.09	Arrawarra	<LOD	<LOD

24	RF 33	11/02/2021	1030	931	7	Arrawarra	<LOD	<LOD
25	RF 34	29/04/2021	1200	1092	10.1	Coffs harbour	<LOD	<LOD
26	RF 35	29/04/2021	1080	979	8	Coffs harbour	<LOD	<LOD
27	RF 51	29/04/2021	1100	997	8.15	Coffs harbour	<LOD	<LOD
28	RF 52	29/04/2021	1150	1045	10.3	Coffs harbour	<LOD	<LOD
29	RF 53	29/04/2021	1120	1016	10.25	Coffs harbour	<LOD	<LOD
30	RF 54	29/04/2021	1150	1045	9.4	Coffs harbour	<LOD	<LOD
31	RF 55	29/04/2021	1150	1045	10.2	Coffs harbour	<LOD	<LOD
32	Fish 6	*na*	780	696	2.8	Sandon Shoals	<LOD	<LOD
33	AG5	29/04/2021	1100	997	8.2	Coffs harbour	<LOD	<LOD
34	MAC 9	16/06/2021	1240	1129	11.44	Hyde Rock reef	<LOD	<LOD
35	MAC14	16/06/2021	1200	1092	9.24	Hyde Rock reef	<LOD	<LOD
36	MAC13	16/06/2021	1150	1045	8.86	Hyde Rock reef	<LOD	<LOD
37	MAC10	16/06/2021	1240	1129	13.14	Wigton island	<LOD	<LOD
38	MAC11	16/06/2021	1050	950	5.76	Wigton island	<LOD	<LOD
39	MAC12	16/06/2021	1050	950	5.52	Calder island	<LOD	<LOD
40	MAC46	17/06/2021	1120	1016	9.8	Wigton island	<LOD	<LOD
41	MAC43	17/06/2021	1200	1092	10.5	Wigton island	<LOD	<LOD
42	MAC44	17/06/2021	1260	1148	12.94	Wigton island	<LOD	<LOD
43	MAC48	17/06/2021	1220	1111	9.24	Wigton island	<LOD	<LOD
44	MAC41	17/06/2021	1150	1045	8.96	Hyde Rock reef	<LOD	<LOD
45	MAC24	17/06/2021	1210	1101	10.89	Wigton island	<LOD	<LOD
46	MAC22	17/06/2021	1210	1101	10.44	Wigton island	<LOD	<LOD
47	MAC47	20/06/2021	1180	1073	8.26	Wigton island	<LOD	<LOD
48	MAC45	2/07/2021	1560	1431	24.06	Derwent Island	<LOD	<LOD
49	MAC42	2/07/2021	1400	1280	15.82	Derwent Island	<LOD	<LOD
50	MAC15	2/07/2021	1200	1092	11	Derwent Island	<LOD	<LOD
51	MAC16	2/07/2021	1260	1148	11.88	Derwent Island	<LOD	<LOD

52	BAG A	2/07/2021	1220	1111	11.14	Derwent Island	<LOD	<LOD
53	BAG B	2/07/2021	1100	997	9.18	Derwent Island	<LOD	<LOD
54	BAG C	2/07/2021	1200	1092	10.4	Derwent Island	<LOD	<LOD
55	REC 470	17/07/2021	1150	1045	8.52	Hyde Rock reef	<LOD	<LOD
56	REC466	17/07/2021	1160	1054	9.02	Hyde Rock reef	<LOD	<LOD
57	REC 407	17/07/2021	1330	1214	14.82	Singapore rock reef	<LOD	<LOD
58	REC 406	17/07/2021	1260	1148	13.2	Heskett rock reef	<LOD	<LOD
59	REC 408	17/07/2021	1260	1148	12.04	Derwent Island	<LOD	<LOD
60	REC 468	17/07/2021	990	894	4.86	Noel island	<LOD	<LOD
61	REC 467	17/07/2021	1050	950	6.82	Bailey island	<LOD	<LOD
62	REC 469	17/07/2021	1170	1063	8.46	Bailey island	<LOD	<LOD
63	REC 452	17/07/2021	1350	1233	16.8	Overfall reef	<LOD	<LOD
64	REC 453	17/07/2021	1230	1120	9.82	Overfall reef	<LOD	<LOD
65	REC 455	17/07/2021	1320	1205	13.7	Prudhoe Island	<LOD	<LOD
66	REC 454	17/07/2021	1220	1111	11.52	Cockermouth island	<LOD	<LOD
67	REC 451	17/07/2021	1190	1082	9.54	Cockermouth island	<LOD	<LOD
68	REC 464	17/07/2021	1290	1177	12.88	Skull rock reef	<LOD	<LOD
69	REC 463	17/07/2021	1150	1045	10.26	Skull rock reef	<LOD	<LOD
70	REC 461	17/07/2021	1200	1092	11.36	Rattray island	<LOD	<LOD
71	REC 462	17/07/2021	1260	1148	10.36	Overfall reef	<LOD	<LOD
72	REC 465	17/07/2021	1140	1035	8.76	Bailey island	<LOD	<LOD
73	REC 425	17/07/2021	1130	1026	8.36	Bailey island	<LOD	<LOD
74	REC424	17/07/2021	1440	1318	16.1	Hyde rock	<LOD	<LOD
75	REC 423	17/07/2021	1230	1120	10.06	Singapore rock reef	<LOD	<LOD
76	REC 422	17/07/2021	1200	1092	9.82	Rattray island	<LOD	<LOD
77	REC 421	17/07/2021	1200	1092	9.34	Rattray island	<LOD	<LOD
78	REC 410	19/07/2021	1220	1111	12.02	Wigton island	<LOD	<LOD

79	REC 449	29/07/2021	1250	1139	11.38	Hyde Rock	<LOD	<LOD
80	REC 450	29/07/2021	1220	1111	10.46	Wigton island	<LOD	<LOD
81	REC 445	24/08/2021	1100	997	7.1	Payne shoal	<LOD	<LOD
82	REC 442	29/08/2021	1000	903	6	Payne shoal	<LOD	<LOD
83	REC 441	29/08/2021	1180	1073	11.9	Payne shoal	<LOD	<LOD
84	REC 459	12/09/2021	1000	903	6	Payne shoal	<LOD	<LOD
85	REC 460	12/09/2021	1000	903	6	Payne shoal	<LOD	<LOD
86	BAL bag 1	10/02/2021	1140	1035	9.6	Brunswick heads	<LOD	<LOD
87	CF box C bag 1	3/02/2021	1120	1016	12	Ballina	<LOD	<LOD
88	CF box C bag 3	12/01/2021	1180	1073	11.7	Ballina	<LOD	<LOD
89	CF box C bag 6	17/02/2021	1300	1186	14.2	Ballina	<LOD	<LOD
90	CF box C bag 15	20/01/2021	1200	1092	10.2	Ballina	<LOD	<LOD
91	CF box C bag 16	4/01/2021	1150	1045	11.7	Ballina	<LOD	<LOD
92	CF box C bag 20	12/01/2021	1150	1045	11	Ballina	<LOD	<LOD
93	CF box C bag 25	17/01/2021	1190	1082	10.8	Ballina	<LOD	<LOD
94	REC 511	16/07/2021	1510	1384	*na*	Fraser Inshore	<LOD	<LOD
95	REC 536	20/07/2021	1310	1196	*na*	Fraser Inshore	<LOD	<LOD
96	REC 537	8/08/2021	1560	1431	*na*	Fraser Inshore	<LOD	<LOD
97	REC 538	8/08/2021	1200	1092	*na*	Fraser Inshore	<LOD	<LOD
98	REC 539	8/08/2021	1280	1167	*na*	Fraser Inshore	<LOD	<LOD
99	REC 544	8/08/2021	1180	1073	*na*	Fraser Inshore	<LOD	<LOD
100	REC 545	8/08/2021	1250	1139	*na*	Fraser Inshore	<LOD	<LOD
101	REC 546	8/08/2021	1420	1299	*na*	Fraser Inshore	<LOD	<LOD

15 References

Adams, M.J. (1993): An outbreak of ciguatera poisoning in a group of scuba-divers. − Journal of Wilderness Medicine 4: 304-311.

Australian Food Regulation Secretariat: Key Documents That Underpin the Joint Food Regulation System (https://foodregulation.gov.au/internet/fr/publishing.nsf/Content/key-system-documents, Page last updated: 02 March 2021)

Bagnis, R., Kuberski, T., Laugier, S. (1979): Clinical observations on 3,009 cases of ciguatera (fish poisoning) in the South Pacific. − The American Journal of Tropical Medicine and Hygiene 28: 1067.

Ballagh, A.C., Begg, G.A., Mapleston, A., Tobin, A. (2006): Growth trends of Queensland east coast Spanish mackerel (*Scomberomorus commerson*) from otolith back-calculations. − Marine and Freshwater Research 57: 383-393.

Beatty, P., Boulter, M., Carter, S., Chinain, M., Doblin, M., Farrell, H., Gatti, C., Hallegraeff, G., Harwood, T., Sandberg, S., et al. (2019) National Ciguatera Research Strategy: Reducing the Incidence of Ciguatera in Australia through Improved Risk Management, Seger, A., Dowsett, N., Turnbull, A., Eds., South Australian Research and Development Institute: Adelaide, Australia 32pp. (https://www.safefish.com.au/-/media/fish-safefish/documents/technical-reports/national-ciguatera-fish- poisoning-research-strategy-final.ashx)

Berdalet, E., Tester, P.A., Zingone, A. (2012): Global Ecology and Oceanography of Harmful Algal Blooms. GEOHAB Core Research Project: HABs in Benthic Systems. In, p. 66. − GEOHAB 2012, Paris and Newark.

Bienfang, P., DeFelice, S., Dowling, A. (2011): Quantitative evaluation of commercially available test kit for ciguatera in fish. − Food and Nutrition Science 2: 594-598.

Boisier, P., Ranaivoson, G., Rasolofonirina, N., Andriamahefazafy, B., Roux, J., Chanteau, S., Satake, M., Yasumoto, T. (1995): Fatal mass poisoning in Madagascar following ingestion of a shark (*Carcharhinus leucas*) - clinical and epidemiologic aspects and isolation of toxins. − Toxicon 33: 1359- 1364.

Caillaud, A., De La Iglesia, P., Darius, H.T., Pauillac, S., Aligizaki, K., Fraga, S., Chinain, M., Diogene, J. (2010): Update on Methodologies Available for Ciguatoxin Determination: Perspectives to Confront the Onset of Ciguatera Fish Poisoning in Europe. − Marine Drugs 8: 1838-1907.

Campàs, M., Leonardo, S., Oshiro, N., Kuniyoshi, K., Tsumuraya, T., Hirama, M., Diogène, J. (2022): A Smartphone-based Portable Biosensor to Assess Ciguatoxin in Fish from the Pacific Ocean. − Food Chemistry 374: 131687.

Campbell, A.B., O'Neill, M.F., Staunton-Smith, J., Atfield, J., Kirkwood, J. (2012): Stock Assessment of the Australian East Coast Spanish Mackerel (*Scomberomorus commerson*) Fishery (The State of Queensland, Department of Agriculture, Fisheries and Forestry).

Campora, C.E., Dierking, J., Tamaru, C.S., Hokama, Y., Vincent, D. (2008b): Detection of Ciguatoxin in fish Tissue using Sandwich ELISA and Neuroblastoma Cell Bioassay. − Journal of Clinical Laboratory Analysis 22: 246-253.

Campora, C.E., Hokama, Y., Tamaru, C.S., Anderson, B., Vincent, D. (2010): Evaluating the Risk of Ciguatera Fish Poisoning from Reef Fish Grown at Marine Aquaculture Facilities in Hawai'i. − Journal of the World Aquaculture Society 41: 61-70.

Campora, C.E., Hokama, Y., Yabusaki, K., Isobe, M. (2008a): Development of an Enzyme-linked Immunosorbent Assay for the Detection of Ciguatoxin in Fish Tissue Using Chicken Immunoglobulin Y. − Journal of Clinical Laboratory Analysis 22: 239-245. http://doi.org/10.1002/jcla.20256

Champion, C., Brodie, S., Coleman, M.A. (2021): Climate-Driven Range Shifts Are Rapid Yet Variable Among Recreationally Important Coastal-Pelagic Fishes. − Frontiers in Marine Science 8: 622299.

Champion, C., Coleman, M.A. (2021): Seascape Topography Slows Predicted Range Shifts in Fish Under Climate Change. − Limnology and Oceanography Letters 6: 143-153.

Champion, C., Hobday, A.J., Zhang, X., Coleman, M.A. (2022): Climate Change Alters the Temporal Persistence of Coastal-Pelagic Fishes Off Eastern Australia. − ICES Journal of Marine Science 79:1083-1097.

Chan, W.H., Mak, Y.L., Wu, J.J., Jin, L., Sit, W.H., Lam, J.C.W., de Mitcheson, Y.S., Chan, L.L., Lam, P.K.S., Murphy, M.B., (2011): Spatial Distribution of Ciguateric Fish in The Republic of Kiribati. − Chemosphere 84(1), 117-123.

Chinain, M., Darius, H.T., Ung, A., Cruchet, P., Wang, Z., Ponton, D., Laurent, D., Pauillac, S. (2010): Growth and Toxin Production in the Ciguatera-Causing Dinoflagellate *Gambierdiscus polynesiensis* (Dinophyceae) in Culture. − Toxicon 56: 739-750.

Chinain, M., Faust, M.A., Pauillac, S. (1999): 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 35: 1282-1296.

Chinain, M., Gatti Howell, C., Roué, M., Ung, A., Henry, K., Revel, T., Cruchet, P., Viallon, J., Darius, H.T. (2023): Ciguatera poisoning in French Polynesia: A review of the distribution and toxicity of *Gambierdiscus* spp., and related impacts on food web components and human health. − Harmful Algae 129

Chinain, M., Germain, M., Sako, Y., Pauillac, S., Legrand, A.-M. (1997): Intraspecific Variation in the Dinoflagellate *Gambierdiscus toxicus* (Dinophyceae) Isozyme Analysis. − Journal of Phycology 33: 36- 43.

Cruz-Rivera, E., Villareal, T.A. (2006): Macroalgal Palatability and the Flux of Ciguatera Toxins Through Marine Food Webs. − Harmful Algae 5: 497-525.

Darius, H., Drescher, O., Ponton, D., Pawlowiez, R., Laurent, D., Dewailly, E., Chinain, M., (2013): 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 30(3), 550-566.

Darius, H., Ponton, D., Revel, T., Cruchet, P., Ung, A., Tchou Fouc, M., Chinain, M. (2007): Ciguatera Risk Assessment in Two Toxic Sites of French Polynesia Using the Receptor-Binding Assay. − Toxicon 50: 612-626.

Dechraoui, M.Y.B., Tiedeken, J.A., Persad, R., Wang, Z., Granade, H.R., Dickey, R.W., Ramsdell, J.S. (2005): Use of Two Detection Methods to Discriminate Ciguatoxins from Brevetoxins: Application to Great Barracuda from Florida Keys. − Toxicon 46: 261-270.

Dechraoui, M.Y.B., Wang, Z., Ramsdell, J.S. (2007): Optimization of Ciguatoxin Extraction Method from Blood for Pacific Ciguatoxin (P-CTX-1). − Toxicon 49: 100-105.

Dickey, R. (2008): Ciguatera Toxins: Chemistry, Toxicology, and Detection. In Seafood and Freshwater Toxins: Pharmacology, Physiology and Detection (L. M. Botana editor), pp. 479-496. − CRC Press, NEW YORK.

Dickey, R.W., Plakas, S.M. (2010): Ciguatera: A public health perspective. − Toxicon 56: 123-136.

Diogène, J., Reverte, L., Rambla-Alegre, M., Del Río, V., de la Iglesia, P., Campàs, M., Palacios, O., Flores, C., Caixach, J., Ralijaona, C., Razanajatovo, I., Pirog, A., Magalon, H., Arnich, N., Turquet, J. (2017): Identification of Ciguatoxins in a Shark Involved in a Fatal Food Poisoning in the Indian Ocean. − Scientific Reports 7:8240.

Edwards, A., Zammit, A., Farrell, H. (2019): Four Recent Ciguatera Fish Poisoning Incidents in New South Wales, Australia Linked to Imported Fish. Communicable Disease Intelligence 43.

EFSA, 2010. Panel on Contaminants in the Food Chain Scientific Opinion on Marine Biotoxins in Shellfish– Emerging toxins: Ciguatoxin Group. − EFSA Journal 8: 1627.

Endean, R., Griffith, J.K., Robins, J.J., Llewellyn, L.E., Monks, S.A. (1993): Variation in the Toxins Present in Ciguateric Narrow-Barred Spanish Mackerel, *Scomberomorus commerson*. − Toxicon 31: 723-732.

FAO and WHO (2020). Report of the Expert Meeting on Ciguatera Poisoning. Rome, 19-23 November 2018. Food Safety and Quality No. 9. Rome.

FAO and WHO (2023). Codex Alimentarius Commission Procedural Manual. Twenty-eighth edition, revised. Rome.

Farrell, H, Murray, S.A., Zammit, A., Edwards, A.W. (2017): Management of Ciguatoxin Risk in Eastern Australia. Toxins 9 :367.

Farrell, H., Zammit, A., Harwood, D.T., Murray, S. (2016b): Is Ciguatera Moving South in Australia? − Harmful Algal News 54: 5-6.

Farrell, H., Zammit, A., Harwood, T., McNabb, P., Shadbolt, C., Manning, J., Turahui, J.A., van den Berg, D.J., Szabo, L. (2016a): Clinical Diagnosis and Chemical Confirmation of Ciguatera Fish Poisoning in New South Wales, Australia. − Communicable Disease Intelligence, 40: E1-E6.

Fauvelot, C., Borsa, P. (2011): Patterns of Genetic Isolation in A Widely Distributed Pelagic Fish, the Narrow-Barred Spanish Mackerel (*Scomberomorus commerson*). − Biological Journal of the Linnean Society 104: 886-902.

Fleming, L.E., Baden, D.G., Bean, J.A., Weisman, R., Blythe, D.G. (1998): Seafood Toxin Diseases: Issues in Epidemiology and Community Outreach. In Harmful Algae (B. Reguera, J. Blanco, M. L. Fernandez, T. Wyatt editors), pp. 245-248. − Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO.

Fraga, S., Rodriguez, F., Caillaud, A., Diogene, J., Raho, N., Zapata, M. (2011): *Gambierdiscus excentricus* sp. nov. (Dinophyceae), a benthic toxic dinoflagellate from the Canary Islands (NE Atlantic Ocean). − Harmful Algae 11: 10-22.

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. (2017): An Updated Review of Ciguatera Fish

Poisoning: Clinical, Epidemiological, Environmental, and Public Health Management. Marine Drugs 15: 72.

Friedman, M.A., Fleming, L.E., Fernandez, M., Bienfang, P., Schrank, K., Dickey, R., Bottein, M.Y., Backer, L., et al. (2008): Ciguatera Fish Poisoning: Treatment, Prevention and Management. − Marine Drugs 6: 456-479.

FSANZ (2006). Safe Seafood Australia. Guide to Standard 4.2.1 Primary Production and Processing Standard for Seafood, Chapter 4 of the Australia New Zealand Food Standards Code (Australia only) Second Edition 130pp.

FSANZ (2023). Australia New Zealand Food Standards Code. (https://www.foodstandards.gov.au/publications/documents/Safe%20Seafood%202edn-WEBwc%20.pdf)

Gaboriau, M., Ponton, D., Darius, H. T. Chinain, M. (2014): Ciguatera Fish Toxicity in French Polynesia: Size Does Not Always Matter. − Toxicon 84: 41-50.

Gillespie, N.C. (1987): Possible Origins of Ciguatera. In Toxic Plants and Animals, a Guide for Australia (J. Covacevich, P. Davie, J. Pearn editors), pp. 171-179. Queensland Museum, Brisbane.

Gillespie, N.C., Lewis, R.J., Pearn, J.H., Bourke, A.T., Holmes, M.J., Bourke, J.B., Shields, W.J. (1986): Ciguatera in Australia. Occurrence, Clinical Features, Pathophysiology and Management. − Medical Journal of Australia 145: 584-590.

Glaziou, P., Legrand, A.M. (1994): The Epidemiology of Ciguatera Fish Poisoning. − Toxicon 32: 863- 873.

Glaziou, P., Martin, P. (1993): Study of Factors That Influence the Clinical Response to Ciguatera Fish Poisoning. − Toxicon 31: 1151-1154.

Granade, H., Cheng, P.C., Doorenbos, N.J. (1976): Ciguatera I: Brine Shrimp (*Artemia salina*) Larval Assay for Ciguatera Toxins. − Journal of Pharmaceutical Sciences 65: 1414-1415.

Hamilton, B., Hurbungs, M., Jones, A., Lewis, R.J. (2002a): Multiple Ciguatoxins Present in Indian Ocean Reef Fish. − Toxicon 40: 1347-1353.

Hamilton, B., Hurbungs, M., Vernoux, J.P., Jones, A., Lewis, R.J. (2002b): Isolation and Characterisation of Indian Ocean Ciguatoxin. − Toxicon 40: 685-693.

Hamilton, B., Whittle, N., Shaw, G., Eaglesham, G., Moore, M.R., Lewis, R.J. (2010): Human Fatality Associated with Pacific Ciguatoxin Contaminated Fish. − Toxicon 56: 668-673.

Hardison, D.R., Holland, W.C., Darius, H.T., Chinain, M., Tester, P.A., Shea, D., Bogdanoff, A.K., Morris Jr, J.A., Flores Quintana, H.A., Loeffler, C.R., (2018): Investigation of Ciguatoxins In Invasive Lionfish from The Greater Caribbean Region: Implications for Fishery Development. − PLOS One 13: e0198358.

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. (2016): Fluorescent Receptor Binding Assay for Detecting Ciguatoxins In Fish. − PLOS One 11: e0153348.

Harwood, D., Murray, S., Boundy, M., (2017): Chapter Three–Sample Preparation Prior to Marine Toxin Analysis. Comprehensive Analytical Chemistry; Diogène, J., Campàs, M., Eds, 89-136.

Hirama, M., Oishi, T., Uehara, H., Inoue, M., Maruyama, M., Guri, H., Satake, M. (2001): Total Synthesis of Ciguatoxin CTX3C. − Science 294: 1904-1907.

Hoffman, P.A., Granade, H.R., Mcmillan, J.P. (1983): The Mouse Ciguatoxin Bioassay - A Dose-Response Curve and Symptomatology Analysis. − Toxicon 21: 363-369.

Hokama, Y. (1985): A Rapid, Simplified Enzyme Immunoassay Stick Test for The Detection of Ciguatoxin And Related Polyethers From Fish Tissues. − Toxicon 23: 939-946.

Hokama, Y. (1990): Simplified Solid-Phase Immunobead Assay for Detection of Ciguatoxin And Related Polyethers. − Journal of Clinical Laboratory Analysis 4: 213-217.

Hokama, Y., Abad, M.A., Kimura, L.H. (1983): A Rapid Enzyme-Immunoassay for The Detection of Ciguatoxin In Contaminated Fish Tissues. − Toxicon 21: 817-824.

Hokama, Y., Banner, A.H., Boylan, D.B. (1977): Radioimmunoassay for detection of ciguatoxin. − Toxicon 15: 317-325.

Holland, W.C., Litaker, R.W., Tomas, C.R., Kibler, S.R., Place, A.R., Davenport, E.D., Tester, P.A. (2013): Differences in The Toxicity of Six *Gambierdiscus* (Dinophyceae) Species Measured Using An In Vitro Human Erythrocyte Lysis Assay. − Toxicon 65: 15-33.

Holmes, M.J. (1998): *Gambierdiscus yasumotoi* sp. nov. (dinophyceae), A Toxic Benthic Dinoflagellate from Southeastern Asia. − Journal of Phycology 34: 661-668.

Holmes, M.J., Venables, B., Lewis, R.J. (2021): Critical Review and Conceptual and Quantitative Models for The Transfer and Depuration of Ciguatoxins in Fishes. − Toxins 13: 515.

Hossen, V., Soliño, L., Leroy, P., David, E., Velge, P., Dragacci, S., Krys, S., Quintana, H.F., Diogène, J., (2015): Contribution to The Risk Characterization of Ciguatoxins: LOAEL Estimated from Eight Ciguatera Fish Poisoning Events in Guadeloupe (French West Indies). − Environmental Research 143: 100-108.

Ikehara, T., Kuniyoshi, K., Oshiro, N., Yasumoto T. (2017): Biooxidation of Ciguatoxins Leads to Species-Specific Toxin Profiles. − Toxins 9: 205.

IOC-UNESCO. (2015): IOC/IPHAB Global Ciguatera Strategy 2015-2019. In, pp. 1-12. − 12th Session of UNESCO-IOC's Intergovernmental Panel on Harmful Algal Blooms, Paris.

Kirk, M., Ford, L., Glass, K., Hall, G. (2014): Foodborne illness, Australia, circa 2000 and circa 2010. − Emerging Infectious Diseases 20: 1857-1864.

Kohli G.S., Haslauer K., Sarowar C., Kretzschmar A.L., Boulter M., Harwood D.T., Laczka O., Murray S.A. (2017): 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 4: 328-334

Kohli, G.S., Farrell, H., Murray, S.A. (2015): *Gambierdiscus*, The Cause of Ciguatera Fish Poisoning: An Increased Human Health Threat Influenced by Climate Change. In Climate Change and Marine and Freshwater Toxins (L. M. Botana, M. C. Louzao, N. Vilarino editors), pp. 271-310. − DE Gruyter, Berlin.

Kohli, G.S., Harwood, D.T., Laczka, O., Boulter, M., Murray, S.A., (2016): Safeguarding Seafood Consumers in New South Wales from Ciguatera Fish Poisoning. FRDC project no. 2014-35.

Kohli, G.S., Papiol, G.G., Rhodes, L.L., Harwood, D.T., Selwood, A., Jerrett, A., Murray, S.A., Neilan, B.A. (2014): A Feeding Study to Probe the Uptake of Maitotoxin By Snapper (*Pagrus auratus*). − Harmful Algae 37: 125-132.

Labrousse, H., Matile, L. (1996): Toxicological Biotest On Diptera Larvae to Detect Ciguatoxins And Various Other Toxic Substances. − Toxicon 34: 881-891.

Langstreth, J., Martin, N., Sanunders, T., Molony, B. (2014): Spanish Mackerel *Scomberomorus commerson*. In Status of Key Australian Fish Stocks Reports Canberra (M. Flood, I. Stobuzki, J. Andrews, C. Ashby, G. Begg, R. Fletcher, C. Gardner, L. Georgeson, S. Hansen, K. Hartmann editors), pp. 461-469. − Fisheries Research and Development Corporation, Australia, Canberra.

Larson, E., Rothman, L. (1967): Ciguatera Poisoning by The Horse-Eye Jack, *Caranx latus*, A Carangid Fish from The Tropical Atlantic. − Toxicon 5: 121-124.

Legrand, A.M., Litaudon, M., Genthon, G.N., Bagnis, R., Yasumoto, T. (1989): Isolation and Some Properties of Ciguatoxin. − Journal of Applied Phycology 1: 183-188.

Legrand, A.-M., Teai, T., Cruchet, P., Satake, M., Murata, K., Yasumoto, T. (1998): Two Structural Types of Ciguatoxin Involved in Ciguatera Fish Poisoning in French Polynesia. In Harmful Algae (B. Reguera, J. Blanco, M. L. Fernandez, T. Wyatt editors), pp. 473-475. − Xunta de Galicia and IOC/UNESCO.

Lehane, L., Lewis, R.J. (2000): Ciguatera: Recent Advances but The Risk Remains. − International Journal of Food Microbiology 61: 91-125.

Leonardo, S., Gaiani, G., Tsumuraya, T., Hirama, M., Turquet, J., Sagristà, N., Rambla-Alegre M., Flores, C., Caixach, J., Diogène, J. (2020): Addressing the Analytical Challenges for The Detection of Ciguatoxins Using an Electrochemical Biosensor. Analytical Chemistry 92: 4858-4865.

Lewis, R., Molgo, J., Adams, D.J. (2000): Ciguatera toxins: Pharmacology of toxins involved in ciguatera and related fish poisonings. In Seafood and Freshwater Toxins: Pharmacology, Physiology and Detection (L. M. BOTANA editor), p. 419. − CRC Press, New York.

Lewis, R.J. (2001): The Changing Face of Ciguatera. − Toxicon 39: 97-106.

Lewis, R.J., Endean, R. (1984): Ciguatoxin From the Flesh and Viscera of The Barracuda, *Sphyraena jello*. − Toxicon 22: 805-810.

Lewis, R.J., Holmes, M.J. (1993): Origin and Transfer of Toxins Involved in Ciguatera. − Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology 106: 615-628.

Lewis, R.J., Inserra, M., Vetter, I., Holland, W.C., Hardison, D.R., Tester, P.A., Litaker, R.W. (2016): Rapid Extraction and Identification of Maitotoxin And Ciguatoxin-Like Toxins from Caribbean And Pacific *Gambierdiscus* Using A New Functional Bioassay. − PLOS One: e0160006.

Lewis, R.J., Jones, A. (1997): Characterization of Ciguatoxins And Ciguatoxin Congeners Present in Ciguateric Fish by Gradient Reversed-Phase High-Performance Liquid Chromatography/Mass Spectrometry. − Toxicon 35: 159-168.

Lewis, R.J., Sellin, M. (1992): Multiple Ciguatoxins In the Flesh of Fish. − Toxicon 30: 915-919.

Lewis, R.J., Sellin, M., Poli, M.A., Norton, R.S., Macleod, J.K., Sheil, M.M. (1991): Purification and Characterization of Ciguatoxins From Moray Eel (*Lycodontis javanicus*, Muraenidae). − Toxicon 29: 1115-1127.

Lewis, R.J., Vernoux, J.P., Brereton, I.M. (1998): Structure of Caribbean Ciguatoxin Isolated from *Caranx latus*. − Journal of the American Chemical Society 120: 5914-5920.

Lewis, R.J., Wong Hoy, A.W., Mcgiffin, D.C. (1992): Action of Ciguatoxin On Human Atrial Trabeculae. − Toxicon 30: 907-914.

Lewis, R.J., Yang, A., Jones, A. (2009): Rapid Extraction Combined With LC-Tandem Mass Spectrometry (CREM-LC/MS/MS) For the Determination of Ciguatoxins In Ciguateric Fish Flesh. − Toxicon 54: 62-66.

Lindsay, J.A. (1997): Chronic Sequelae of Foodborne Disease. − Emerging Infectious Diseases 3: 443.

Litaker, R.W., Hardison, D.R., Holland, W.C., Bourdelais, A.J., McCall, J.R., Baden, D.G., Morris Jr, J.A., Bogdanoff, A.K., Tester, P.A. (2014): Ciguatoxin Concentrations in Invasive Lionfish Estimated Using A Fluorescent Receptor Binding Assay. Marine and Fresh-Water Harmful Algae 184.

Littler, M.M., Littler, D.S. (1980): The Evolution Of Thallus Form And Survival Strategies In Benthic Marine Macroalgae: Field And Laboratory Tests Of A Functional Form Model. The American Naturalist 116: 25–44

Lucas, R.E., Lewis, R.J., Taylor, J.M. (1997): Pacific Ciguatoxin-1 Associated with A Large Common- Source Outbreak of Ciguatera in East Arnhem Land, Australia. − Natural Toxins 5: 136-140.

Mackie, M., Buckworth, R.C., Gaughan, D.J. (2003): Stock Assessment of Narrow-Barred Spanish Mackerel (*Scomberomorus commerson*) in Western Australia. Technical report Perth, Western Australia: Department of Fisheries.

Mak, Y.L., Wu, J.J., Chan, W.H., Murphy, M.B., Lam, J.C., Chan, L.L., Lam, P.K. (2013): Simultaneous Quantification of Pacific Ciguatoxins in Fish Blood Using Liquid Chromatography–Tandem Mass Spectrometry. − Analytical and Bioanalytical Chemistry 405: 3331-3340.

Manger, R.L., Leja, L.S., Lee, S.Y., Hungerford, J.M., Wekell, M.M. (1993): Tetrazolium-Based Cell Bioassay for Neurotoxins Active on Voltage-Sensitive Sodium Channels: Semiautomated Assay for Saxitoxins, Brevetoxins, And Ciguatoxins. Analytical Biochemistry 214: 190-194.

Mattei, C., Dechraoui, M.Y., Molgo, J., Meunier, F.A., Legrand, A.M., Benoit, E. (1999): Neurotoxins Targeting Receptor Site 5 of Voltage-Dependent Sodium Channels Increase the Nodal Volume of Myelinated Axons. − Journal of Neuroscience Research 55: 666-673.

McPherson GR (2007) Historical stock definition research on *Scomberomorus commerson* in Queensland waters pp 30-60, In Buckworth, R, Newman, S, Ovenden, J, Lester, R and McPherson, G 2007, The stock structure of northern and western Australian Spanish Mackerel, Fishery report 88, final report, Fisheries Research and Development Corporation Project 1998/159 (Fisheries Group, Northern Territory Department of Business, Industry and Resource Development, Darwin).

McPherson, G.R. (1981): Identification of Stocks and Migration Routes of Spanish Mackerel. FIRTA (Fishing Industry Research Trust Account) Report.

McPherson, G.R. (1992): Age And Growth Of The Narrow-Barred Spanish Mackerel (*Scomberomorus commerson* Lacepede, 1800) in North-Eastern Queensland Waters. − Australian Journal of Marine and Freshwater Research 43: 1269–1282.

Meyer, L., Carter, S., Capper, A. (2015): An Updated Ciguatoxin Extraction Method and Silica Cleanup for Use With HPLC-MS/MS for The Analysis Of P-CTX-1, P52-epi-54-deoxy-CTX-1B (formerly known as CTX-2) and P-CTX-3. − Toxicon 108: 249-256.

Murata, M., Legrand, A.M., Ishibashi, Y., Fukui, M., Yasumoto, T. (1990): 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 112: 4380- 4386.

Murata, M., Legrand, A.M., Ishibashi, Y., Yasumoto, T. (1989): Structures of Ciguatoxin And Its Congener. − Journal of the American Chemical Society 111: 8929-8931.

Murphy, J.J., Ochwada-Doyle, F.A., West, L.D., Stark, K.E., Hughes, J.M., Taylor, M.D. (2022): Survey of Recreational Fishing in NSW, 2019/20 – Key Results. NSW DPI – Fisheries Final Report Series No. 161 (NSW Department of Primary Industries).

Murphy, J.J., Ochwada-Doyle, F.A., West, L.D., Stark, K.E., Hughes, J.M., (2020): The NSW Recreational Fisheries Monitoring Program - Survey of Recreational Fishing, 2017/18, NSW DPI - Fisheries Final Report Series No. 158 (NSW Department of Primary Industries).

Murray, J.S., Boundy, M.J., Selwood, A.I., Harwood, D.T. (2018): 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 80: 80-87

NSW DPI (2021) NSW DPI Game Fish Tagging Program: Report 2020-2021 (https://www.dpi.nsw.gov.au/ data/assets/pdf_file/0007/1414663/GFT-Report-2020-21.pdf)

NSW Food Authority (2017). (https://www.foodauthority.nsw.gov.au/consumer/food-poisoning/fish-ciguatera-poisoning)

Oshiro, N., Yogi, K., Asato, S., Sasaki, T., Tamanaha, K., Hirama, M., Yasumoto, T., Inafuku, Y. (2010): Ciguatera incidence and fish toxicity in Okinawa, Japan. − Toxicon 56: 656-661.

O'Toole, A.C., Bottein, M.-Y.D., Danylchuk, A.J., Ramsdell, J.S., Cooke, S.J., 2012. 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 427: 98-105.

Ovenden JR, Street R (2007) Genetic population structure of Spanish Mackerel. In R Buckworth, S Newman, JR Ovenden, RJ Lester and G McPherson (eds), The stock structure of Northern and

Pasinszki, T., Lako, J., Dennis, T.E., 2020. Advances in Detecting Ciguatoxins in Fish. Toxins 12: 494.

Poli, M.A., Lewis, R.J., Dickey, R.W., Musser, S.M., Buckner, C.A., Carpenter, L.G. (1997): Identification of Caribbean ciguatoxins as the cause of an outbreak of fish poisoning among US soldiers in Haiti. − Toxicon 35: 733-741.

Pottier, I., Hamilton, B., Jones, A., Lewis, R.J., Vernoux, J.P. (2003): Identification of slow and fast acting toxins in a highly ciguatoxic barracuda (Sphyraena barracuda) by HPLC/MS and radiolabelled ligand binding. − Toxicon 42: 663-672.

Pottier, I., Vernoux, J.P., Jones, A., Lewis, R.J. (2002): Characterisation of multiple Caribbean ciguatoxins and congeners in individual specimens of horse-eye jack (Caranx latus) by high-performance liquid chromatography/mass spectrometry. − Toxicon 40: 929-939.

QLD Government (2022) East coast Spanish mackerel fishery harvest strategy: 2023–2028 Consultation Draft (Qld Department of Agriculture and Fisheries, Brisbane).

Queensland Health. (2015): Communicable disease control guidance and information. (http://disease-control.health.qld.gov.au/, accessed 10/1/16.)

Randall, J.E. (1958): A review of ciguatera, tropical fish poisoning, with a tentative explanation of its cause. − Bulletin of Marine Science 8: 236-267.

Rhodes, L.L., Smith, K.F., Munday, R., Selwood, A.I., Mcnabb, P.S., Holland, P.T., Bottein, M.-Y. (2010): Toxic Dinoflagellates (Dinophyceae) from Rarotonga, Cook Islands. − Toxicon 56: 751-758.

Ridgway, K.R., 2007. Long-term trend and decadal variability of the southward penetration of the East Australian Current. Geophysical Research Letters, 34(13).

Rowling, K., Hegarty, A., Ives, M. (2010): Status of Fisheries Resources in NW 2008/09. In, p. 392. − Industry & Investment NSW, Cronulla, Australia.

SafeFish (2019): National Ciguatera Research Strategy: Reducing the Incidence of Ciguatera in Australia Through Improved Risk Management. (https://www.safefish.com.au/reports/technical-reports/national-ciguatera-fish-poisoning-research-strategy)

SAFS (Status of Australian Fish Stocks) (2020): Spanish Mackerel (https://fish.gov.au/report/393-Spanish-Mackerel-2020#:~:text=The%20latest%20stock%20assessment%20model,2020%5D).

Sanchez-Henao, J.A., García-Álvarez, N., Fernández, A., Saavedra, P., Sergent, F.S., Padilla, D., Acosta-Hernández, B., Suárez, M.M., Diogène, J., Real, F. (2019): 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 673: 576-584.

Satake, M., Fukui, M., Legrand, A.M., Cruchet, P., Yasumoto, T. (1998): Isolation and Structures of New Ciguatoxin Analogs, 2,3-Dihydroxyctx3c and 51-Hydroxyctx3c, Accumulated in Tropical Reef Fish. − Tetrahedron Letters 39: 1197-1198.

Satake, M., Ishibashi, Y., Legrand, A.M., Yasumoto, T. (1996): Isolation and Structure of Ciguatoxin- 4A, a New Ciguatoxin Precursor, from Cultures of Dinoflagellate Gambierdiscus toxicus and Parrotfish *Scarus gibbus*. − Bioscience, Biotechnology, and Biochemistry 60: 2103-2105

Satake, M., Murata, M., Yasumoto, T. (1993): The Structure of CTX3C, A Ciguatoxin Congener Isolated from Cultured *Gambierdiscus toxicus*. − Tetrahedron Letters 34: 1975-1978.

Scheuer, P.J., Takahashi, W., Tsutsumi, J., Yoshida, T. (1967): Ciguatoxin: Isolation and Chemical Nature. − Science 155: 1267-1268.

SFM (2015): Schedule of Ciguatera High-Risk Areas and Species Size Limits.

SFM (Sydney Fish Market) (2015): Seafood Handling Guidelines. (https://www.sydneyfishmarket.com.au/Portals/0/adam/Content/41UIctIuJECV0p4vxMVS4Q/ButtonLink/Seafood%20Handling%20Guidelines.pdf)

Sims, J.K. (1987): A Theoretical Discourse on The Pharmacology of Toxic Marine Ingestions. − Annals of Emergency Medicine 16: 1006.

Skinner, M.P., Brewer, T.D., Johnstone, R., Fleming, L.E., Lewis, R.J. (2011): Ciguatera Fish Poisoning in the Pacific Islands (1998 to 2008). − PLoS Neglected tropical diseases 5: e1416.

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.

Steneck, R.S., Dethier, M.N. (1994):. A Functional Group Approach to The Structure of Algal-Dominated Communities. − Oikos 69: 476–498

Stewart J, Hegarty A, Young C, Fowler AM, Craig J (2015): Status of Fisheries Resources in NSW 2013–14 (NSW Department of Primary Industries, Mosman).

Stewart, I., Lewis, R.J., Eaglesham, G., Graham, G., Poole, S., Craig, S. (2010): Emerging Tropical Diseases in Australia. Part 2. Ciguatera Fish Poisoning. − Annals of Tropical Medicine and Parasitology 104: 557-571.

Sulaiman, Z.H., Ovenden, J.R. (2010): Population Genetic Evidence for The East–West Division of The Narrow-Barred Spanish Mackerel (*Scomberomorus commerson*, Perciformes: Teleostei) Along Wallace's Line. − Biodiversity and Conservation 19: 563-574.

Sumner, J. (2011): Hazards Affecting Australian Seafood Part 1: Priority Listing of Issues and Risk Ranking of Hazards Affecting Australian Seafood and Part 2: Supporting Information (Sumner, 2011). Report to SafeFish and the Australian Seafood Cooperative Research Centre (May, 2011). SARDI, Urrbrae, SA.

Suthers, I.M., Young, J.W., Baird, M.E., Roughan, M., Everett, J.D., Brassington, G.B., Byrne, M., Condie, S.A., Hartog, J.R., Hassler, C.S. and Hobday, A.J., 2011. The strengthening East Australian Current, its eddies and biological effects—an introduction and overview. Deep Sea Research Part II: Topical Studies in Oceanography, 58(5), pp.538-546.

Szabo, E.A., Arundell, E.J., Farrell, H. Imlay, A., King, T., Shadbolt, C., Taylor. M.D (2022): Responding to Incidents of Low-Level Chemical Contamination in Food. In Martinovic, A., Oh, S., Lelieveld, H. Ensuring Global Food Safety 2nd Edition Exploring Global Harmonization, pp. 359-377.

Tanimoto, M., Fox, A.R., O'Neill, M.F., Langstreth, J. (2020): Stock Assessment of Australian East Coast Spanish Mackerel (*Scomberomorus commerson*). Qld Dept Agriculture and Fisheries, Brisbane.

Ting, J., Brown, A. (2001): Ciguatera Poisoning: A Global Issue with Common Management Problems. − European Journal of Emergency Medicine 8: 295-300.

Tobin, A., Heupel, M., Simpfendorfer, C., Buckley, S., Thurstan, R., Pandolfi, J. (2014): Utilising Innovative Technology to Better Understand Spanish Mackerel Spawning Aggregations and The Protection Offered by Marine Protected Areas (Centre for Sustainable Tropical Fisheries and Aquaculture, James Cook University, Townsville).

Tobin, A., Mapleston, A. (2004): Exploitation Dynamics and Biological Characteristics of The Queensland East Coast Spanish Mackerel (*Scomberomorus commerson*) Fishery. CRC Reef Research Centre Technical Report No 51 (CRC Reef Research Centre, Townsville).

Tsumuraya, T., Hirama, M. (2019): Rationally Designed Synthetic Haptens To Generate Anticiguatoxin Monoclonal Antibodies, And Development of a Practical Sandwich ELISA to Detect Ciguatoxins. − Toxins 11: 533.

Tsumuraya, T., Sato, T., Hirama, M., Fujii, I. (2018): Highly Sensitive and Practical Fluorescent Sandwich ELISA for Ciguatoxins. − Analytical Chemistry 90: 7318-7324.

USFDA (2011): Fish and Fishery Products Hazards and Controls Guidance. Fourth Edition, April 2011. In, p. 15. − U.S. Department of Health and Human Services, Food and Drug Administration.

Vernoux, J., Lahlou, N., Magras, L., Greaux, J., (1985): Chick Feeding Test: A Simple System to Detect Ciguatoxin. − Acta tropica 42: 235-240.

Vernoux, J.P., Lewis, R.J. (1997): Isolation and characterisation of Caribbean ciguatoxins from the horse-eye jack (*Caranx latus*). − Toxicon 35: 889-900.

Viallon, J., Chinain, M., Darius, H.T. (2020): Revisiting the Neuroblastoma Cell-Based Assay (CBA- N2a) For the Improved Detection of Marine Toxins Active on Voltage Gated Sodium Channels (Vgscs). − Toxins 12: 281.

Ward, R.D., Zemlak, T.S., Innes, B.H., Last, P.R., Hebert, P.D. (2005): DNA Barcoding Australia's Fish Species. − Philosophical Transactions of the Royal Society of London B: Biological Sciences 360: 1847-1857.

Welch, D.J., Courtney, T., Harry, A., Lawson, E, Moore, B.R., Tobin, A., Turnbull, C., Vance, D., Williams, A.J. (2014): Implications of Climate Change Impacts on Fisheries Resources of Northern Australia (James Cook University, Townsville).

Welch, D.J., Hoyle, S.D., McPherson, G.R., Gribble, N.A. (2002): Preliminary Assessment of The Queensland East Coast Spanish Mackerel Fishery. Department of Primary Industries, Queensland. Information Series QI02110 (The State of Queensland, Department of Primary Industries, Brisbane).

Western Australian Spanish Mackerel, Fishery Report 88 (Fisheries Research and Development Corporation project 98/159, Northern Territory Government, Darwin).

WHO, 2020. Report of The Expert Meeting on Ciguatera Poisoning: Rome, 19-23 November 2018. Food & Agriculture Org.

Williams, R., Lester, R. (2006): Stock Structure of Spanish Mackerel *Scomberomorus commerson* Along the Australian East Coast Deduced from Parasite Data. − Journal of Fish Biology 68: 1707-1712.

Wong, C.K., Hung, P., Lee, K.L.H., Kam, K.M. (2005): Study of An Outbreak of Ciguatera Fish Poisoning in Hong Kong. − Toxicon 46: 563-571.

Wong, C.K., Hung, P., Lee, K.L.H., Mok, T., Chung, T., Kam, K.M. (2008): Features of Ciguatera Fish Poisoning Cases in Hong Kong 2004-2007. − Biomedical and Environmental Sciences 21: 521-527.

Yasumoto, T. (2005): Chemistry, Etiology, And Food Chain Dynamics of Marine Toxins. − Proceedings of the Japan Academy Series B-Physical and Biological Sciences 81: 43-51.

Yasumoto, T., Igarashi, T., Legrand, A.M., Cruchet, P., Chinain, M., Fujita, T., Naoki, H. (2000): Structural Elucidation of Ciguatoxin Congeners by Fast-Atom Bombardment Tandem Mass Spectroscopy. − Journal of the American Chemical Society 122: 4988-4989.

Yasumoto, T., Scheuer, P.J., (1969): Marine Toxins of The Pacific—VIII Ciguatoxin From Moray Eel Livers. − Toxicon 7(4), 273-276.

Yogi, K., Oshiro, N., Inafuku, Y., Hirama, M., Yasumoto, T. (2011): Detailed LC-MS/MS Analysis of Ciguatoxins Revealing Distinct Regional and Species Characteristics in Fish and Causative Alga from The Pacific. − Analytical Chemistry 83: 8886-8891.

Yogi, K., Sakugawa, S., Oshiro, N., Ikehara, T., Sugiyama, K., Yasumoto, T., (2014). Determination of Toxins Involved in Ciguatera Fish Poisoning in The Pacific by LC/MS. − Journal of AOAC International 97: 398-402.