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Safeguarding seafood consumers in New South Wales from ciguatera fish poisoning

Scomberomorus commerson

Spanish Mackerel

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UNIVERSITY OF TECHNOLOGY SYDNEY

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2 Acknowledgments

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3 Abbreviations

CFP	Ciguatera Fish 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 bio assay
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
OHFSS	Queensland health forensic and Scientific Services
LC-MS	Liquid chromatography mass spectrometry
WA	Western Australia

4 Executive Summary

Ciguatera Fish Poisoning (CFP) is a well-known illness in tropical regions, which occurs through the consumption of fish that have accumulated naturally occurring toxins. Ciguatoxins (CTXs) are produced by benthic dinoflagellates of the genus *Gambierdiscus spp*, which are found in warm marine waters. CTXs are cyclic polyether toxins that accumulate through the food chain and can cause CFP, even when CTX levels in fish are very low. In Australia, cases of CFP have occurred due to fish caught in Queensland (QLD), Northern Territory (NT) and New South Wales (NSW) waters. Spanish Mackerel (*Scomberomorus commerson*) is the principal fish species that has resulted in CFP cases from fish caught in NSW and sub-tropical Queensland waters.

CTXs are odourless and tasteless, therefore it is difficult to distinguish toxic fish from those that are harmless. As scientific knowledge of CFP worldwide is relatively low, no validated monitoring methods or measurement methods are available. The only feasible prevention methods used internationally currently are to avoid the consumption of larger fish of certain fish species, avoid certain fish species altogether, or avoid fish caught from certain regions. The objectives of the present study were to: 1) Set up a facility to determine P-CTX-1B presence in fish in NSW; 2) Determine whether CTX could be found in Spanish Mackerel from NSW, and 3) If found, examine the relationship, if any, between the size (length and weight) of *S. commerson* and the incidence of detectable P-CTX1B toxin. Our final objective was to liase with industry regarding the results of this study.

From 71 Spanish Mackerel collected in NSW from Forster (~32° S), to the QLD border, the region in which almost all of Spanish Mackerel in NSW are caught, liver and flesh tissues from one fish, and liver tissues from 4 other fish were positive for P-CTX1B. A difference in toxin content in flesh and liver tissues was found, such that liver tissues had a significantly higher concentration (~ 6 times) of P-CTX-1B than flesh tissues. From 13 fish specimens collected in QLD, liver and flesh tissues from 5 fish and flesh from 1 other fish specimen were positive for P-CTX1B. Toxin levels found were 0.13 µg kg^{-1} to <0.1 µg kg^{-1} in flesh samples, and 1.39 µg kg^{-1} to <0.4 µg kg^{-1} in liver samples.

In the total dataset examined in this report we also included data from three *S. commerson* specimens from northern NSW caught during 2014, which were implicated in incidences of CFP, giving 87 fish samples in the total dataset. These were compared in order to examine the relationship between length and weight of the fish and incidences of detectable P-CTX1B toxin. No apparent relationship could be observed between the length or weight of Spanish Mackerel and the detection of P-CTX1B.

4.1 Keywords

Spanish Mackerel, *Scomberomorus commerson,* ciguatera fish poisoning, ciguatoxins, fish length, toxicology, LC-MS

5 Introduction

5.1 Ciguatera Fish Poisoning

CFP is recognised as one of two major safety risks for Australian seafood products (Sumner, 2011), and is the most common non-bacterial illness associated with fish consumption internationally, (Friedman *et al.*, 2008), affecting between 50,000 and 500,000 people per year (Fleming *et al.*, 1998). The ingestion of herbivorous and carnivorous fish that have orally accumulated effective levels of CTXs is the cause of CFP (Bagnis *et al.*, 1979; Gillespie, 1987; Sims, 1987). Despite being significantly underreported, CFP occurrence worldwide is increasing, with reports of a 60% increase in CFP in the Pacific region over the last decade (Skinner *et al.*, 2011).

Species of the genus *Gambierdiscus* are the main producers of CTXs (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). The role of CTXs in CFP is well established. These toxins accumulate in the food web when toxic *Gambierdiscus* cells attached to surfaces, for example macroalgae, are consumed by herbivorous fish, which are then preyed on by carnivorous fish (Figure 1). The toxins are thought to undergo several oxidation steps during this passage through fish digestive systems, which may increase their potency (Murata *et al.*, 1990a; Lewis *et al.*, 1991; Lewis & Holmes, 1993; Yasumoto *et al.*, 2000). Several CTX congeners have been isolated from the viscera-digestive organs, liver and muscle of carnivorous 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).

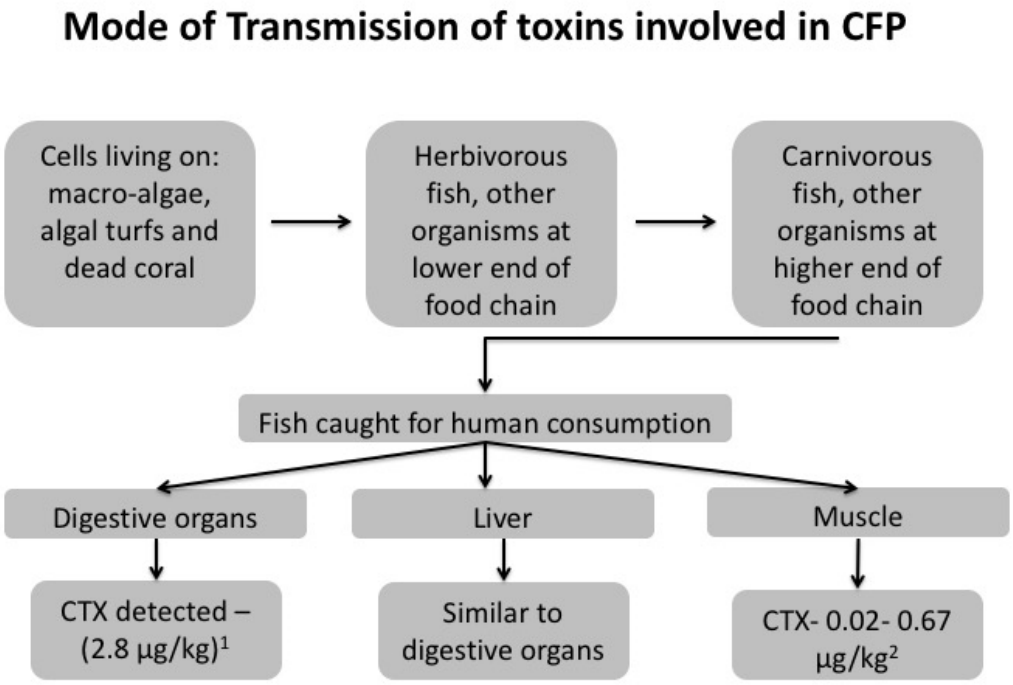


Figure 1: Flow chart showing the mode of transmission of Ciguatoxins in ciguatera fish poisoning (Kohli *et al.*, 2014, Legrand *et al.*, 1989, Lewis & Sellin, 1992)}.

CTXs are sodium channel activators, particularly affecting the voltage sensitive channels located along the nodes of Ranvier (peripheral nerve cells) (Lewis *et al.*, 1992; Mattei *et al.*, 1999; Lewis *et al.*, 2000). When the sodium channels are activated there is a massive influx of Na^+ ions, resulting in cell depolarisation (Lewis *et al.*, 1992; Mattei *et al.*, 1999; Lewis *et al.*, 2000). This leads to the onset of spontaneous action potentials in effected cells, causing various symptoms in humans. There is a high likelihood of misdiagnosis for CFP. The number of documented symptoms, which are in excess of 175 (Sims, 1987), may vary depending on portion size (Wong *et al.*, 2008), individual susceptibility or accumulation of toxin with age (Bagnis *et al.*, 1979; Glaziou & Martin, 1993) and could also be associated with other illnesses (e.g. decompression sickness (Adams, 1993), chronic fatigue syndrome, multiple sclerosis (Lindsay, 1997; Ting & Brown, 2001) and brain tumours (Lindsay, 1997)). Symptoms can include but are not limited to gastrointestinal, neurological and sometimes cardiovascular, in cases of severe intoxication (Sims, 1987) and can vary depending on geographical region (Lewis *et al.*, 2000; Dickey, 2008). This can be due to the structural differences of CTXs in different regions, therefore it is very important to characterise CTXs from Pacific, Caribbean and the Indian Oceans. Local understanding of CTX accumulation patterns in different fish species can also help prevent CFP. However, the accurate identification of exact congeners of CTXs is necessary, in order to understand the toxicology and evaluate the local risks of CFP.

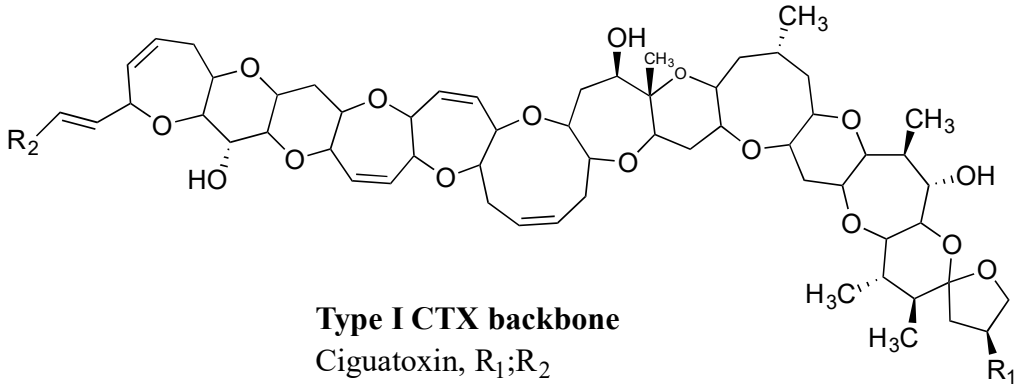
5.1.1 Chemistry of CTXs

Structurally, CTXs are thermostable, cyclic polyether ladders, which are liposoluble (Figure 2). They have been isolated from fish and different species of *Gambierdiscus* (Table 1). Based on their origin and differences in the structure of these toxins, they are divided into P-CTXs (Pacific Ocean), C-CTXs (Caribbean region) and I-CTXs (Indian Ocean). Due to their structural differences, P-CTXs are further divided into type I and type II (Legrand *et al.*, 1998). Type I P-CTXs have 13 rings and 60 carbon atoms (Murata *et al.*, 1990a; Lewis *et al.*, 1991; Lewis & Holmes, 1993; Yasumoto *et al.*, 2000). This category consists of the first CTX to be fully structurally described as CTX1B (Murata *et al.*, 1990a) (or CTX-1 as described by Lewis et al. 1991) from moray eels, which is the principal toxin in the carnivorous fish from the Pacific (Murata *et al.*, 1990b; Lewis *et al.*, 1991).

Two other type I P-CTXs i.e. CTX-2 and CTX-3 were also described from the same extracts, which have slight variations in their structures leading to different toxicities in mice (Lewis *et al.*, 1991) (Table 1). CTX-1, CTX-2 and CTX-3 may be derived from dinoflagellate ciguatoxins CTX-4A and CTX4B (also named as GTX-4B in (Murata *et al.*, 1990b)) (Lewis & Holmes, 1993; Yasumoto *et al.*, 2000). CTX-4A and CTX-4B have been isolated from *G. polynesiensis* culture extracts (Chinain *et al.*, 2010). CTX3C is a type II P-CTX with 13 rings, 57 carbon atoms and was first isolated from cultures of *Gambierdiscus* sp. (Satake *et al.*, 1993) and later from *G. polynesiensis* (Chinain *et al.*, 2010). Two more congeners of CTX3C called as 49-epi-CTX-3C (also called as CTX-3B in (Chinain *et al.*, 2010)) and M-seco-CTX-3C have also been isolated from *Gambierdiscus* sp. (Satake *et al.*, 1993) and *G. polynesiensis* (Chinain *et al.*, 2010). Later, 2 new type II P-CTXs i.e. 2,3 dihydroxyCTX3C (also called as CTX2-A1) and 51-hydroxyCTX3C were isolated from Moray eel (Satake *et al.*, 1998) that might be oxygenated metabolites of CTX3C (Yasumoto *et al.*, 2000).

Caribbean CTXs are slightly bigger than P-CTXs and have 14 rings and 62 carbon atoms (Vernoux & Lewis, 1997; Lewis *et al.*, 1998; Pottier *et al.*, 2002; Pottier *et al.*, 2003). Many congeners of C-CTXs have been isolated from carnivorous fish, which includes C-CTX1, C-CTX-2, C-CTX-1141, C-CTX-1127, C-CTX-1143, C-CTX-1157, C-CTX-1159 (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* sp. 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. Recently 4 CTXs (I-CTX-1, I-CTX-2, I-CTX-3, I-CTX-4) have been isolated from carnivorous fish from the Indian Ocean and have higher molecular ion masses than P-CTXs and C-CTXs (Hamilton *et al.*, 2002a; Hamilton *et al.*, 2002b; Caillaud *et al.*, 2010). However, their structures need to be elucidated (Hamilton *et al.*, 2002a; Hamilton *et al.*, 2002b). I-CTX-1 is toxic to mice via intraperitoneal injection (Hamilton *et al.*, 2002b). Based on Mouse bioassays (MBA), different

congeners of CTXs can have variable toxicities (Table 1), however this needs to be further validated as well.



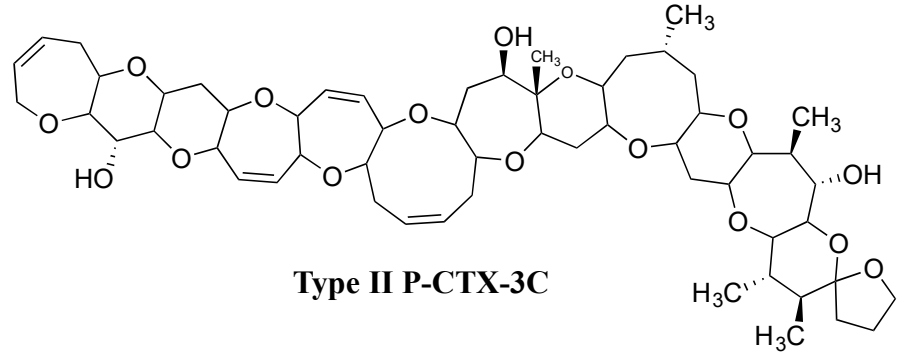
Type I CTX backbone

Ciguatoxin, R_1;R_2

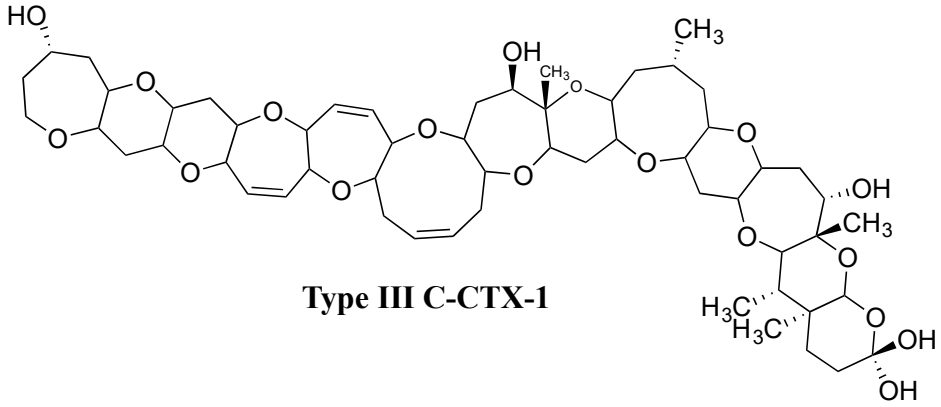
P-CTX-1, OH; $CH(OH)CH_2OH$

P-CTX-2, H; $CH(OH)CH_2OH$

P-CTX-4B, H; $CH=CH_2$



Type II P-CTX-3C



Type III C-CTX-1

Figure 2: Structure of Ciguatoxins (CTXs). P-CTX-1, P-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).

Table 1: The known congeners of CTXs and MTXs, 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)
	CTX-2	1095.5(Lewis *et al.*, 1991)	Giant Moray (*Gymnothorax javanicus*) (Lewis *et al.*, 1991)	2.3 µg/kg (Lewis *et al.*, 1991)
	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

5.1.2 Detection of CTXs in Seafood

The CTX-positive cases are predominantly from the mid-latitude tropical and sub-tropical zones. This is in accordance with the distribution of *Gambierdiscus* (Kohli *et al.,* 2015 and references therein). However, CFP has also been reported in non-endemic areas because of an increase in seafood imports (Glaziou & Legrand, 1994; Ting & Brown, 2001). While the majority of studies have focused on reef fish, toxin accumulation has been observed in eels, sea cucumbers, starfish, seals and jellyfish (Kohli *et al.,* 2015 and references therein). Sharks have also been suspected of causing CFP following outbreaks of human illness, however, remnant samples for testing were unavailable to validate this (Boisier *et al.*, 1995; Lehane & Lewis, 2000). Further studies are required to address the deficit in information for species other than fish and to identify potential toxin vectors in coastal systems. .

Often, in small island nations, local people are aware of ciguatera prone zones and avoid certain fish species. Such knowledge certainly has its merits, however, a study (Darius *et al.*, 2007) in French Polynesia demonstrated the presence of CTXs in fish species that were considered safe to eat by locals. Experimentally, CTX toxin profiles and structures have been determined by chromatographic techniques (HPLC, UPLC and LC-MS), accompanied by nuclear magnetic resonance (NMR) (Murata *et al.*, 1989; Murata *et al.*, 1990a; Lewis *et al.*, 1991; Satake *et al.*, 1996) and radio ligand binding (RLB) (Hamilton *et al.*, 2002a; Hamilton *et al.*, 2002b). However, these methods are not commonplace or practical for regular routine testing, as they are costly and require special expertise. Confirmation of toxin by UPLC/HPLC followed by LC-MS involves the isolation and fractionation of CTX compounds, of known molecular weights. Although a slightly faster method for the extraction of samples for CTX analysis has been proposed (Lewis *et al.*, 2009), acquiring purified CTX standards remains problematic due to the limited supply of purified natural CTX compounds (Berdalet *et al.*, 2012). Though artificial synthesis of CTX is possible (Hirama *et al.*, 2001), it is highly complex. Without a consistent source of reference material, absolute quantification of CTXs and their congeners is hard to achieve. Technical issues such as co-eluting peaks of similar compounds and inhibiting/promoting matrix effects remain unresolved unless a known CTX standard is used.

Several biological assays have been developed for the detection of ciguateric fish. These have included the use of chickens (Pottier et al., 2000), cats (Larson & Rothman, 1967), mongooses (Hokama *et al.*, 1977), diptera larva (Labrousse & Matile, 1996), brine shrimp (Granade *et al.*, 1976) and mosquitoes (Bagnis et al., 1987). However, each assay has its own constraints and limitations, largely relating to toxin specificity and quantification but also due to inefficiencies and ethical considerations (summarised in de Fouw, 2001, Dickey & Plakas, 2010). While the MBA by intraperitoneal injection does not provide a linear dose-response relationship with CTX toxicity (Hoffman *et al.*, 1983), it remains the most widely used biological assay. Numerous biochemical assays have been proposed as alternatives to biological assays for testing seafood. The development of a radioimmunoassay (Hokama *et al.*, 1977) progressed to a cheaper alternative enzyme-linked immunosorbent assay (ELISA) with higher throughput (Hokama *et al.*, 1983). The ELISA test has recently shown promising correlations with biological assays (Campora *et al.*, 2008; Campora *et al.*, 2010). Stick enzyme immunoassay (SEIA) (Hokama, 1985) and solid phase immunoassay (SPIA) (Hokama, 1990) tests have led to the development of commercial kits (i.e. Cigua-check ® and Ciguatect®). However, these products have yielded a large number of false positive and false negative results (Wong *et al.*, 2005) and the Cigua-check® test is no longer being manufactured. Other assays utilised for screening CTXs in fish are the sodium channel binding mouse neuroblastoma cells assay (N2A) (Dickey, 2008) and receptor binding assay (RBA) (Poli *et al.*, 1997; Darius *et al.*, 2007). Both of these assays have shown promising results and have been recommended by the European Food Standard Association (EFSA, 2010). These assays cannot quantify specific congeners of CTXs and MTXs. This can only be achieved via further development and validation via LC-MS analysis, and there is an urgent need to do so. The progress has been disadvantaged by the lack of available purified standards (Guzman-Perez and Park, 2000). Other challenges are that more than one type of CTXs can be present in fish specimens (Endean *et al.*, 1993; Vernoux & Lewis, 1997).

Given the technical issues with the available bioassays for detecting and quantifying CTX compounds, in the present study, we decided to use LC-MS analysis with a known and quantified standard for P-CTX-1B. As this had not previously been established in a format for routine testing in NSW, the first objective of this project was to set up a facility with this capability, and to verify the results of testing using an international best-practise laboratory.

5.2 CFP in Australia

Ciguatera is a well-known disease in the warmer waters of Australia along the coastline of Queensland (QLD) and the Northern Territory (NT) to Byron Bay in NSW (~28°S). We found no evidence of confirmed reports of CFP from Western Australia (WA). Most CFP outbreaks have resulted from fish caught in QLD and the NT (eastern Arafura Sea), with most documented cases involving Spanish Mackerel (Gillespie *et al.*, 1986; Farrell *et al.*, 2016). Prior to 2014, almost all cases of CFP in NSW, Victoria or other southern states have been caused by fish from QLD or the NT, or fish imported from other countries (Farrell *et al.*, 2016).

The number of different fish species implicated in CFP outbreaks is suggested to be of the order of several hundred (Halstad, 1978, FAO, 2004) (Kohli *et al.*, 2015). However, with the absence of a reliable, commercially available test kit, it is difficult to determine an exact figure. While most of the fish species implicated are carnivorous, herbivorous species (Randall, 1958; Cruz-Rivera & Villareal, 2006) have also been linked to CFP outbreaks. Table 2 is a list of fish that have been found to accumulate CTXs in Australia. Other carnivorous tropical fish genera such as Amberjack (*Seriola* spp.), Wrasse (*Cheilinus* spp.) and Trevally (*Caranx* spp.) are also common vectors of CTXs in the Pacific region (Lewis, 2001; Stewart *et al.*, 2010).

In NSW, confirmed CFP cases linked to the consumption of Spanish Mackerel caught in NSW waters have been reported from 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) (Farrell *et al.*, 2016). All people were diagnosed with CFP as they suffered classic CFP symptoms (Farrell *et al.*, 2016).

Many of those involved required hospitalisation, and at least one victim was disabled for at least 7 months (Farrell *et al.*, 2016). P-CTX-1B was detected via LC-MS/MS in Spanish Mackerel samples implicated during the three outbreaks, with concentration levels that are discussed later in this report. Other suspected CFP outbreaks in 2005 and 2009 in NSW were linked to fish originally caught from Fiji and QLD respectively (Farrell *et al.*, 2016). These outbreaks were clinically diagnosed as CFP, however no chemical analysis was performed to detect P-CTX-1B in the implicated seafood. The NSW CFP cases in 2014-2015 are the southernmost confirmed sources of CFP in Australia (Farrell *et al.*, 2016).

Table 2: CTXs detected in seafood in Australia and the method of detection.

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 (Lewis & Jones, 1997), (Lewis *et al.*, 1991; Satake *et al.*, 1998)	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), 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)

5.3 The Spanish Mackerel industry in Australia

In Australia, Spanish Mackerel is widely distributed along the east, west and northern coasts. It has been found that there are likely three main biological stocks of Spanish Mackerel across northern Australia, based on genetics (Langstreth *et al.*, 2014). However, these may consist of smaller stocks that rarely interact, based on the analyses of other variables (Langstreth *et al.*, 2014). The use of parasites suggested that there may be six separate stocks of Spanish Mackerel across northern Australia (Williams & Lester, 2006), however the use of isozyme, allozyme and mitochondrial DNA

genetic analysis failed to find any significant differences (Rowling *et al.*, 2010; Sulaiman & Ovenden, 2010; Fauvelot & Borsa, 2011). As it has been difficult to obtain the relevant data to assess these smaller stocks, Spanish Mackerel has been grouped into two biological stocks (Torres Strait and east coast [Queensland]), two management units (Gulf of Carpentaria [Queensland] and Mackerel Managed Fishery [Western Australia]) and one jurisdiction (Northern Territory) (Langstreth *et al.*, 2014).

The east coast biological stock is distributed along the coast of QLD from Cape York to mid NSW (Langstreth *et al.*, 2014). Although permanent resident populations of Spanish Mackerel exist in QLD, some populations disperse after spawning from reefs and can move up to 1000 nautical miles down south, entering NSW waters (Langstreth *et al.*, 2014). Sparse populations of Spanish Mackerel exist along the coast of NSW all the way down to St. Helens, in Tasmania (Rowling *et al.*, 2010). Within the geographical distribution, adult fish are found in rocky shoals, coral reefs and current lines, both on outer reef areas and offshore, while juveniles are found in more sheltered habitats such as estuaries, creeks and mud flats (Rowling *et al.*, 2010). Female fish are partial spawners, highly fecund and can produce up to 1 million eggs (Rowling *et al.*, 2010). They mainly breed in the spring/summer months in QLD (Rowling *et al.*, 2010), and can reach up to 240 cm in length and a maximum weight of 70 kg in QLD (Oldest commercial catch in QLD, male: 127 cm, 19kg; female: 155 cm, 35 kg) (Rowling *et al.*, 2010). Spanish Mackerel are carnivorous, and their diet consists mainly of small pelagic fish, squid and prawns (Rowling *et al.*, 2010).

The annual combined commercial and recreational catch of the east coast biological stock of *S. commerson* can vary between 200-600 tonnes (Rowling *et al.*, 2010; Langstreth *et al.*, 2014). The last reported commercial catch of 260.9 tonnes was in 2013 (Figure 3). Of this, the NSW commercial landings were relatively small in 2013 (< 10 tonnes) (Rowling *et al.*, 2010; Langstreth *et al.*, 2014). The quantity of NSW caught *S. commerson* sold through the Sydney Fish Market (SFM) has increased: from 6.1 tonnes in 2013 to 28 tonnes in the 2015 calendar year (pers comm SFM). The remaining 170-570 tonnes per annum are caught as part of the QLD commercial fishery (Figure 3). In NSW, the recreational catch of *S. commerson* is estimated to be 10-100 tonnes per year (Rowling *et al.*, 2010). Therefore, in NSW, in general more *S. commerson* is caught recreationally compared to commercial catches, by a proportion of ~2 or more. Recreationally and commercially, the vast majority of the *S. commerson* caught in NSW are from northern NSW waters, particularly north of Coffs Harbour. Large recreational fishing clubs which are orientated to deep sea fishing, and with occasional competitions solely dedicated to Spanish Mackerel, exist at Coffs Harbour and Byron Bay.

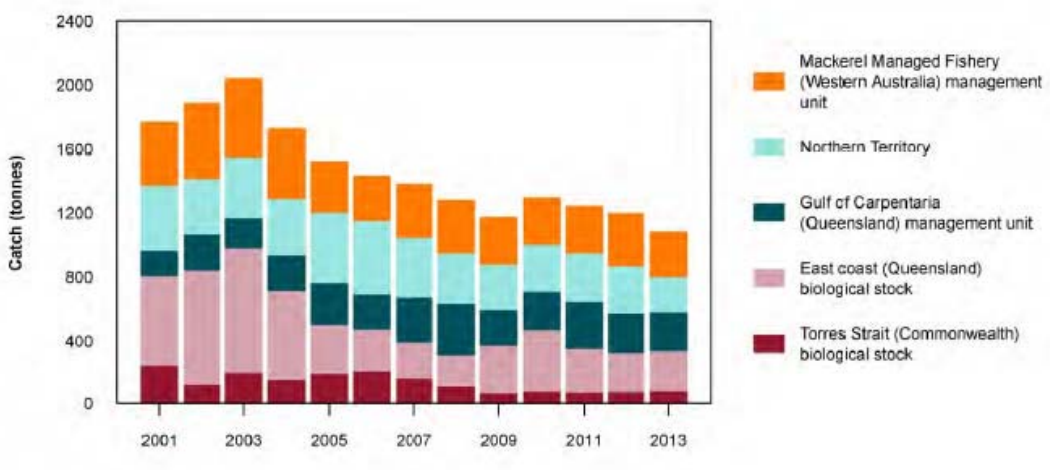


Figure 3: Commercial landings of Spanish Mackerel caught nationally, 2001-2013 (Langstreth *et al.*, 2014).

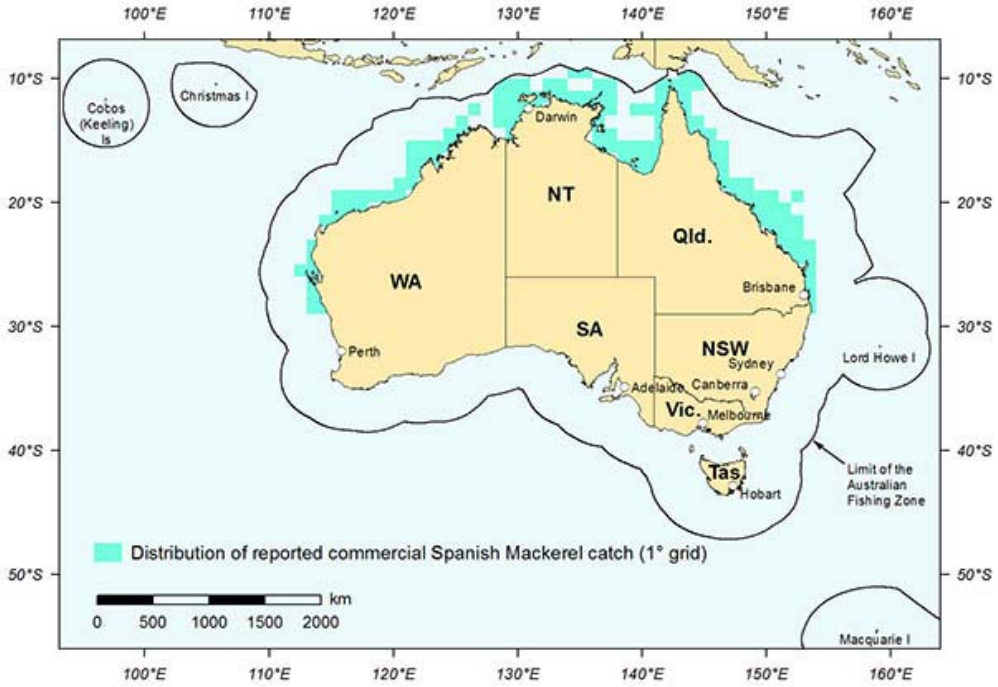


Figure 4: Distribution of reported commercial catch of Spanish Mackerel in Australia (Langstreth *et al.*, 2014).

5.4 Management of CFP

The US Food and Drug Administration (FDA) have published a suggested safe consumption '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). In the absence of rapid, cost-effective and reliable screening tests for CTXs as discussed above, health authorities around the world have mostly provided guidelines to prevent high-risk fish from entering the commercial market to reduce the risk of CFP (Stewart *et al.*, 2010). The general conjecture is that the size or age of fish of certain species are likely to have a relationship with the levels of CTXs found (Stewart *et al.*, 2010). This is likely due to the fact that CTXs can bioaccumulate over time, and therefore older fish could be considered more likely to have higher levels of CTXs.

Relatively few studies have directly examined the evidence for a relationship between fish size and CTX presence. No study has examine the evidence for this relationship in Spanish Mackerel, therefore, it is useful to review the evidence from the few studies that have undertaken this. A study in Japan found a positive relationship of size vs toxicity in specimens of *Lutjanus monostigma* (Onespot Snapper, Figure 5), *Epinephelus fuscoguttatus* (Flowery Rockcod, Figure 6), *Lutjanus bohar* (Red Bass, Figure 7) and *Variola louti* (Yellowedge Coronation Trout, Figure 8) (Oshiro *et al.*, 2010). In total, 612 fish were tested for toxicity equivalent to P-CTX-1B (via MBA) and 108 fish were found to be toxic (*L. monostigma*: 32.3%, *E. fuscoguttatus*: 20.8%, *L. bohar*: 11.9%, *V. louti*: 14.3%) (Oshiro *et al.*, 2010). The study also reports a parallel increase of toxicity and body weight in all four species (Oshiro *et al.*, 2010). P-CTX-1B was detected in all toxic samples, however not quantified (Oshiro *et al.*, 2010). In another study, 40 *Sphyraena barracuda* (Barracuda), liver samples were analysed using a bioassay based on mouse neuroblastoma cells assay (N2A), and 60% of the fish samples were found to be toxic (Dechraoui *et al.*, 2005). The most toxic fish contained 2.1 ppb C-CTX-1 equivalents (Dechraoui *et al.*, 2005). However, no relationship between fish size/weight and toxicity was observed (Figure 9) (Dechraoui *et al.*, 2005).

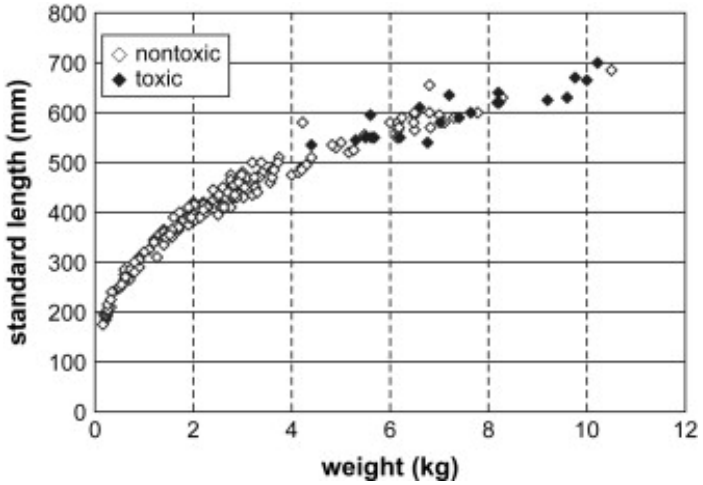


Figure 5: Size of toxic specimens of *L. monostigma* (Onespot Snapper) (Oshiro *et al.*, 2010).

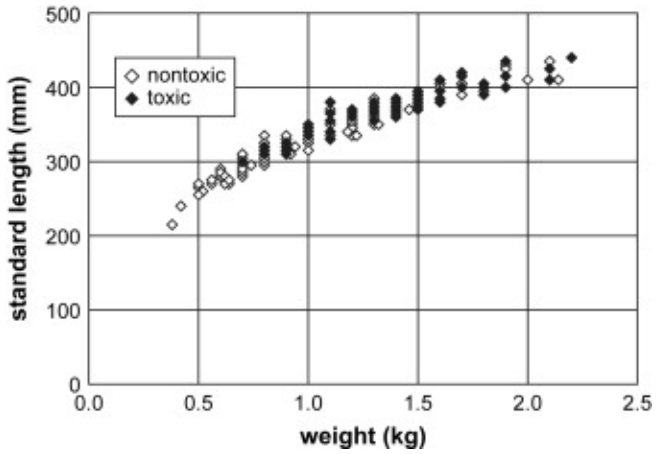


Figure 6: Size of toxic specimens of *E. fuscoguttatus* (Flowery Rockcod, Oshiro *et al.*, 2010).

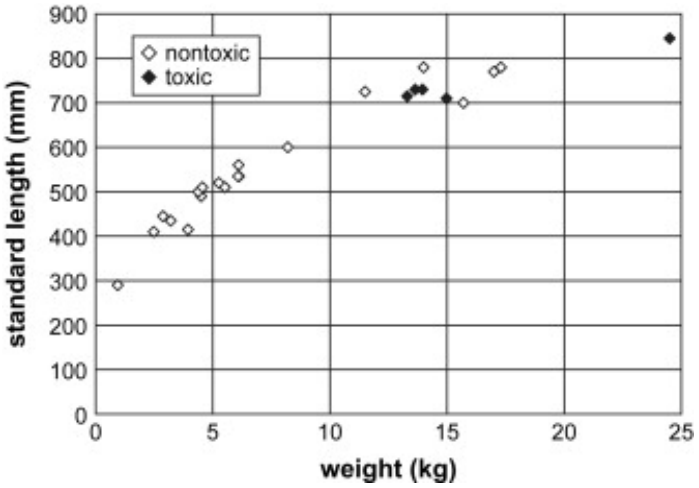


Figure 7: Size dependency of toxic specimens of *L. bohar* (Red Bass, Oshiro *et al.*, 2010).

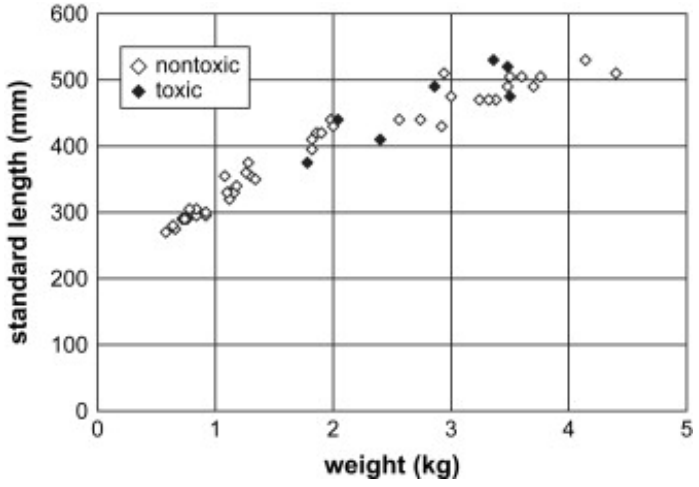


Figure 8: Size dependency of toxic specimens of *V. louti* (Yellowedge Coronation Trout, Oshiro *et al.*, 2010).

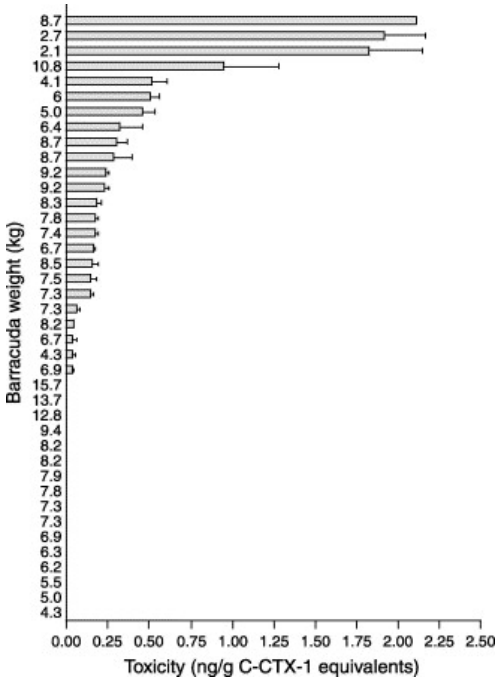


Figure 9: Caribbean ciguatoxin C-CTX-1 equivalents measured in liver specimens of 40 *Sphyraena barracuda (*Barracuda) caught off the coast of Marathon Key, FL, USA by cytotoxicity assay. Each column, assigned with the weight of each fish, represents the mean±SEM (*n*=3 except for the fish weighing 8.7 kg) (Dechraoui *et al.*, 2005).

In summary, in the very few studies that have directly examined the evidence for a relationship between fish size and CTX presence in a fish species anywhere in the world, results have been mixed. While some studies have shown support for this relationship, others have not found evidence of this. To date, no evidence has been collected of the relationship between size and CTX toxins in Spanish Mackerel.

In Australia, The Sydney Fish Markets, (SFM), the largest domestic fish distributor in Australia, provides guidelines to prevent high-risk fish from entering the market (Stewart *et al.*, 2010). Table 3 and

Table 4~~Table 4~~ provide the current schedule of ciguatera high-risk areas and species size limit for sale (SFM, 2015). The QLD and NT authorities also follow these guidelines (QLDHealth, 2015). Additionally, in QLD, CFP outbreaks and any intoxication related to CFP is a notifiable condition under the requirements of the 2005 Public Health Regulation of the Public Health Act 2015 (QLDHealth, 2015). Once notified, QLD Health follows the communicable disease guidelines to recover any remaining fish tissue implicated in CFP and send it for quantification of P-CTX-1, 2 and 3 to QLD health forensic and Scientific Services (OHFSS). Since CFP is a communicable disease in QLD, well-established protocols and procedures are in place to report the disease and collect samples. However, more research is needed to access and mitigate the risk of CFP in Australia.

Table 3: 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 Immediate vicinity of Cape Arnhem North East Island and Connexion Island (both near Groote Eylandt Gove Peninsula, in the immediate vicinity of Nhulunbuy)	The following species: Pickhandle Barracuda (*Sphyraena jello*) Bluespotted Rockcod (*Cephalopholis cyanostigmata*) 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 4: 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

6 Objectives

The objectives of the present study were formulated in consultation with the commercial fishing community, the NSW Food Authority and the Sydney Fish Market. They were:

- Establish the first testing facility for CTXs in NSW.
- Determine if CTXs are present in Spanish Mackerel caught in NSW waters, and if so, generate qualitative and quantitative information.
- If found, analyse data on CTX presence and concentration in relation to: fish size, location that fish was caught, date, water temperature.
- Liaise with commercial fishing organisations and regulators to inform them of the evidence on CTXs and procedures for testing fish.

7 Methods

7.1 Fish sampling

Approximately 400 sampling packs were distributed to the Sydney Fish Market, Byron Bay Deep Sea fishing club, Coffs Harbour Deep Sea Fishing club and the following fishing co-operatives across the Northern NSW coast: Coffs Harbour, Evans Head, Ballina and Brunswick. These clubs and locations were chosen as 1) Recreational fishing for Spanish Mackerel is significant in northern NSW, and may represent up to 90% of the total catch, and 2) The vast majority of the Spanish Mackerel catch in NSW comes from these regions. For comparison, samples packs were also distributed to a recreational fishing group in far Northern QLD. Fish from this region are also considered part of the east coast Spanish Mackerel stock.

The sample pack consisted of several labelled tubes, which could contain ~10 g 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. Sample packs were given out to commercial and recreational fishing groups in northern NSW during January-March 2015, at which time they also received an explanation regarding the project, and several presentations were also given at recreational fishing club meetings. 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.

7.2 Toxin analysis via LC-MS

7.2.1 Toxin Extraction Protocol

7.2.1.1 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.

7.2.1.2 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.

7.2.1.3 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.

7.2.2 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 in Table 5.

Table 5: 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 SIMS the analysis was performed on a Thermo Scientific™ Q EXACTIVE™ high resolution mass-spectrometer equipped with an electrospray ionization source. Chromatographic separation was performed on a Thermo Scientific™ ACCELA™ UPLC system with an injection volume of 5 µL. The following source parameters were used in all experiments: a capillary temperature of 272 ^{0}C, a spray voltage of 3.5 kV, an auxiliary gas heater temperature of 442 ^{0}C, a sheath gas and an auxiliary gas flow rate of 54 and 14 (arbitrary units). The mass spectrometer was operated in the positive mode scanning across the range of *m/z* 500 - 1,500. For detection of P-CTX-1B, a target-mass of *m/z* 1128.6102 was extracted with a mass tolerance of 5 ppm, which is consistent with the ammoniated-adduct ($[M+NH_4]^+$) of the target molecule. This adduct showed the highest and most consistent peaks for the used method in earlier studies. For quantitation, peak areas were integrated and sample concentrations calculated from linear calibration curves generated from standards. Thermo Xcalibur software (version 3.0.63, Thermo Fisher Scientific, Inc.) was used for the analysis.

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).

7.2.3 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.

7.3 Spanish Mackerel identification via qPCR

To determine the identity of fish specimen collected, DNA was extracted from 0.5 g of liver tissue of each specimen via the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). The manufacturer's protocol was followed and samples were stored at -20˚C until PCR amplification.

To determine whether the samples originate from the same species, *S.commerson,* a quantitative PCR was performed. All PCR reactions were performed in 20 µL reaction volumes containing 10 µL SYBR® Select Master Mix (Bioline, Eveleigh, NSW), 10 pmol each of the forward and reverse primers and between 10 and 100 ng genomic DNA. Primer-pair (TGGGCCGTCCTTATTACAGC, CTCCTCCTGCTGGGTCAAAG) specific for the cytochrome oxidase subunit I (COI) gene from *S.commerson,* were used (Ward *et al.*, 2005). Cycling conditions are listed in Table 6.

Table 6: 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

8 Results

8.1 Objective 1

8.1.1 Establishment of LC-MS/MS facility for P-CTX-1B detection

Initial method development to quantify P-CTX-1B in samples was carried out at the Cawthron Institute, Nelson, New Zealand on a Waters Xevo TQ-S triple quadrupole mass spectrometer coupled to a Waters Acquity UPLC i-Class with flow through needle sample manager.

Liquid chromatography conditions developed at Cawthron were transferred to the SIMS Thermo Scientific™ Q EXACTIVE™ high resolution mass-spectrometer coupled with a Thermo Scientific™ ACCELA™ UPLC system.

The instrument was calibrated and mass spectrometer parameters were optimised for P-CTX-1B quantification using standards provide by Prof. Takeshi Yasumoto from Okinawa Science and Technology Promotion Centre, Okinawa, Japan.

The performance of the method established at the SIMS facility was assessed and linear calibration over concentration range tested. The inter and intra day variability in sample quantification was evaluated. Spiking experiments (spiking P-CTX-1B standard in fish liver and flesh samples) were carried out to calculate the toxin recovery rate, variance and standard deviation to monitor potential matrix impacts.

8.1.2 Evaluation of spiked samples & statistic parameters

Table 7: Recovery rates as percentage from the debit.

Sample	Matrix	Retention time [min]	Peak area	Calculated concentration [ng/mL]	Percentage of measured concentration to debit [%][1]
1	Flesh	2.4	46.974	0.93948	55.26
2	Flesh	2.39	15.084	0.30168	17.75
3	Flesh	2.33	22.245	0.4449	26.17
4	Flesh	2.31	12.042	0.24084	14.17
5	Flesh	2.31	72.346	1.44692	85.11
6	Flesh	2.31	3.821	0.07642	4.50
7	Liver	2.38	10.417	0.20834	12.22
8	Liver	ND[2]	ND[2]	NC[3]	NC[3]
9	Liver	2.38	1.796	0.03592	2.11
10	Liver	ND[2]	ND[2]	NC[3]	NC[3]
11	Liver	2.31	57.000	1.14	66.87

[1]debit=1.705ng/mL
[2]ND = not detected,
[3]NC = not calculated

As seen in Table 7, the recovery rates vary significantly between individual samples. In samples 8 and 10, both liver samples, no detectable amount of P-CTX-1B was found. Therefore, a correction of the toxin values was needed. To obtain realistic results, the calculated concentration was corrected using the recovery rate factor, which represents the amount of implemented standard that can be detected on

average after the extraction and measurement. In order to determine the average recovery rate, the equation described in Table 8 was used, obtaining a final value of 25.83%. Therefore, a factor of 3.92 was used to adjust the measured values in the *S.commerson* samples to the real values, calculated in the following way:

Calculation of recovery rate factor (r)

r = 100%/25.83% = 3.92

The variability of the calculated concentration is s^2=0.231684, with a standard deviation of s=0.481336. The variability of the recovery rate is s^2=800.28, the standard deviation is s=28.29. This implies considerable fluctuations of ± 0.48 ng/mL or ± 28.29% for presumed equal conditions resulting from the effect of either the extraction method or the measurement setup.

Table 8: Equations used to calculate statistical parameters.

Parameter	Abbreviation	Equation
Variability	s^2	$s^2 = \frac{\sum_{i=1}^{n}(x-\bar{x})^2}{n}$
Standard deviation	s	$s = \sqrt{s^2}$
Average	$\bar{x}$	$\bar{x} = \frac{\sum_{i=1}^{n} x_i}{n}$

8.2 Objective 2

8.2.1 Detection of P-CTX-1B in *S.commerson* samples

In total, 84 samples of *S. commerson* were collected (71 from NSW and 13 from QLD). Most of the samples returned were from the recreational fishing community, who showed a relatively high level of engagement with this project, amongst many individual participants. The commercial fishing community showed a comparatively lower rate of participation, which was partly due to unforeseen staff shortages at some organisations. Nevertheless, we consider this an overall relatively good rate of return, considering that the sample collection required a high commitment from participants. Samples from Coffs Harbour recreational fishers were the most numerous (Table 9). This reflected the fact that this is the largest and most active recreational fishing community in the area, that Spanish Mackerel are taken in greater numbers by recreational fishers as compared to commercial fishers in NSW (see Page 8), and that this mid-far north coast region is the region from which the vast majority of Spanish Mackerel are caught in NSW (Page 8). As explained earlier, Spanish Mackerel are infrequently caught south of Port Macquarie in NSW, such that the ~400km stretch of coastline from Port Macquarie to the Queensland Border, in which all fish in this study were caught, represents almost the complete region for the Spanish Mackerel fishery in NSW.

From 71 fish specimen collected in NSW, liver and flesh tissues from one fish (Figure 10,

Table 9~~Table 9~~) and liver tissues from 4 other fish specimens were positive for P-CTX1B (Figure 11,

Table 9~~Table 9~~). Whereas, from the 13 fish specimen collected in QLD, liver and flesh tissues from 5 fish and flesh from 1 other fish specimen were positive for P-CTX1B (

Table 9~~Table 9~~).

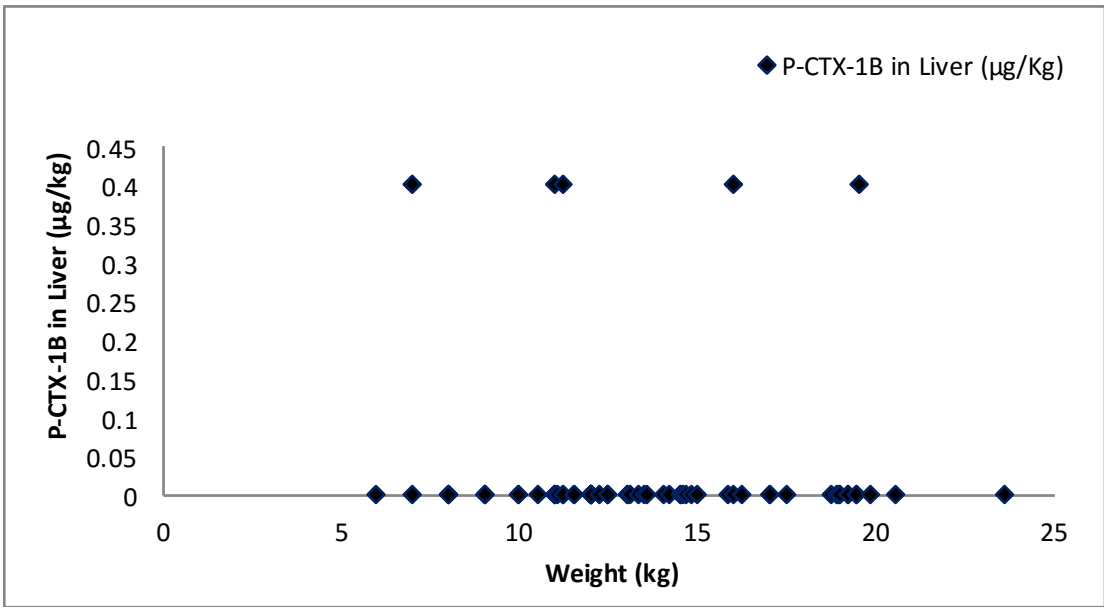


Figure 10: Absolute quantification of P-CTX-1B in liver tissue of fish caught in NSW.

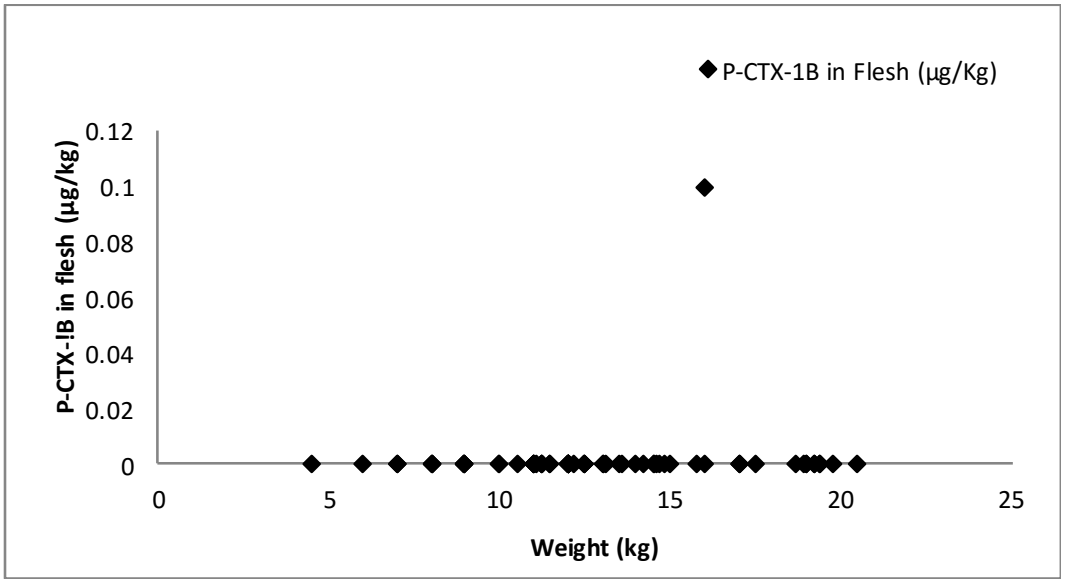


Figure 11: Absolute quantification of P-CTX-1B in flesh tissue of fish caught in NSW.

Table 9: LC-MS analysis of P-CTX-1B in samples of *S. commerson* flesh and liver collected for this study, and from an analysis of fish implicated in poisoning 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°.47.88S 149°.25.18E)	--	136	16	0.13	1.39
AIMS-6	Great Barrier Reef, Rockhampton, QLD (22°.00.48S 152°.38.85E)	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

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]
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	1/04/15	120	14.5	ND	ND

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]
	Harbour, NSW					
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, NSW[2]	2/3/14	--	25.7	0.4	NT
V1207-B	Evans Head, NSW[2]	13/2/14	--	10-17[3]	0.6	NT
V1207-C3	Evans Head, NSW[2]	13/2/14	--	10-17[3]	1.0	NT
V1207-D4	Evans Head, NSW[2]	13/2/14	--	10-17[3]	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.

8.3 Objective 3

8.3.1 Determination of size: toxin content ratio in toxic fish

To determine any measurable relationship between the size of *S.commerson* caught vs the level of P-CTX-1B found in liver and flesh samples, data from fish collected in NSW, QLD and previous 2014-2015 CFP incidents in NSW were pooled together. No noticeable correlation was observed (Figure 12, Figure 13). Although the levels measured in the *S.commerson* samples are quite low, they are higher than the US Food and Drug administration's level considered safe for human consumption (0.01 µg Kg^{-1} CTX equivalent for P-CTX-1B). Figure 14 shows the relationship between the weight and length of toxic/non-toxic specimens of Spanish Mackerel. The graph demonstrates that specimens of toxic Spanish Mackerel are distributed evenly among all size categories. The trend line in the graph represents the mean of the length vs mass relationship of all Spanish Mackerel, demonstrating that toxic specimens of Spanish Mackerel tend to be lighter for their length as compared to the non-toxic specimens of Spanish Mackerel. The reason for this trend are unclear, and may require further analysis.

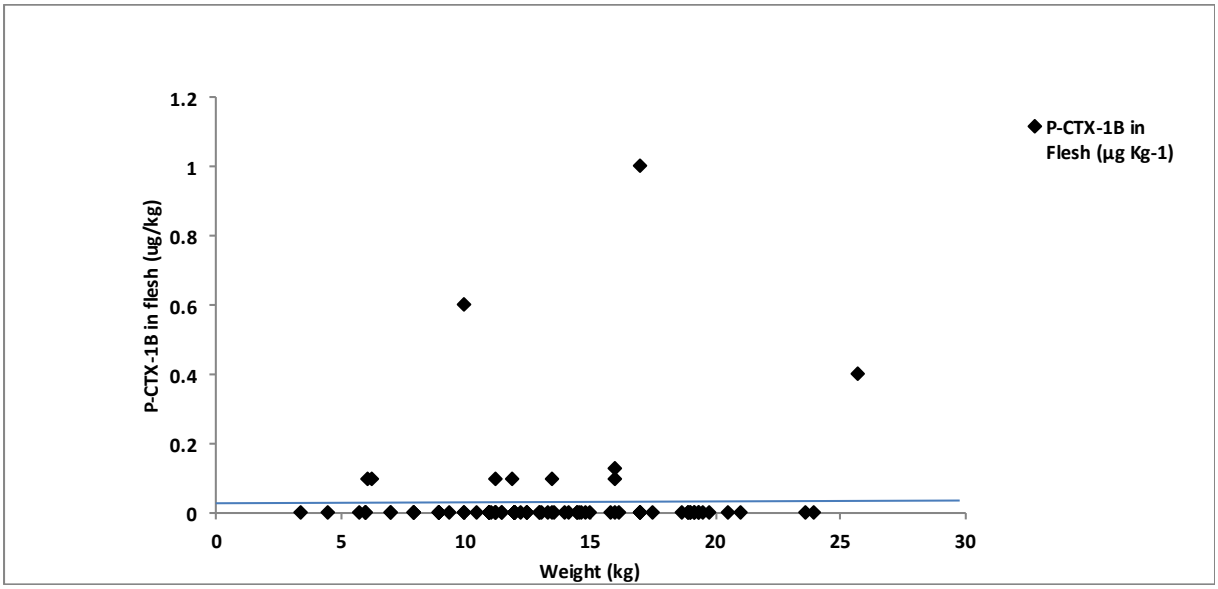


Figure 12: Relationship between the weight and level of P-CTX-1B in flesh tissue of fish caught in NSW, QLD and previous 2014-2015 CFP incidents in NSW (n=87). The blue line represents the US FDA level considered safe for human consumption.

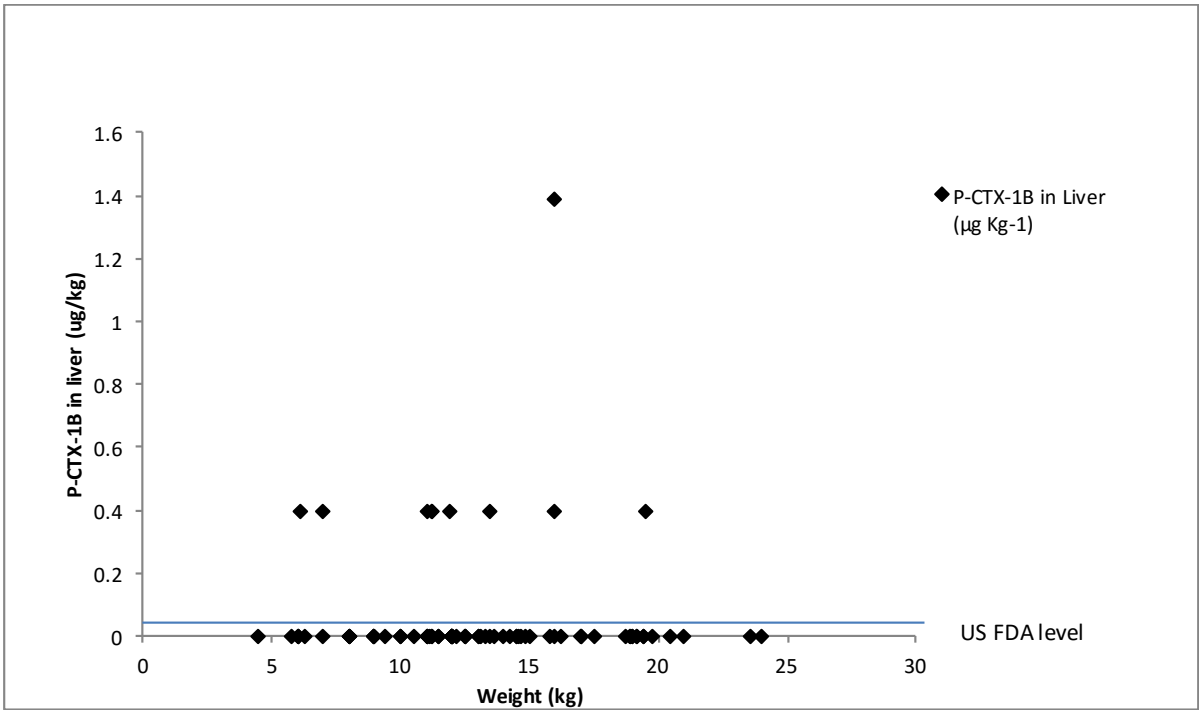


Figure 13: Relationship between the weight and level of P-CTX-1B in liver tissue of fish caught in NSW, QLD and previous 2014-2015 CFP incedents in NSW (n=87). The blue line represents the US FDA level considered safe for human consumption.

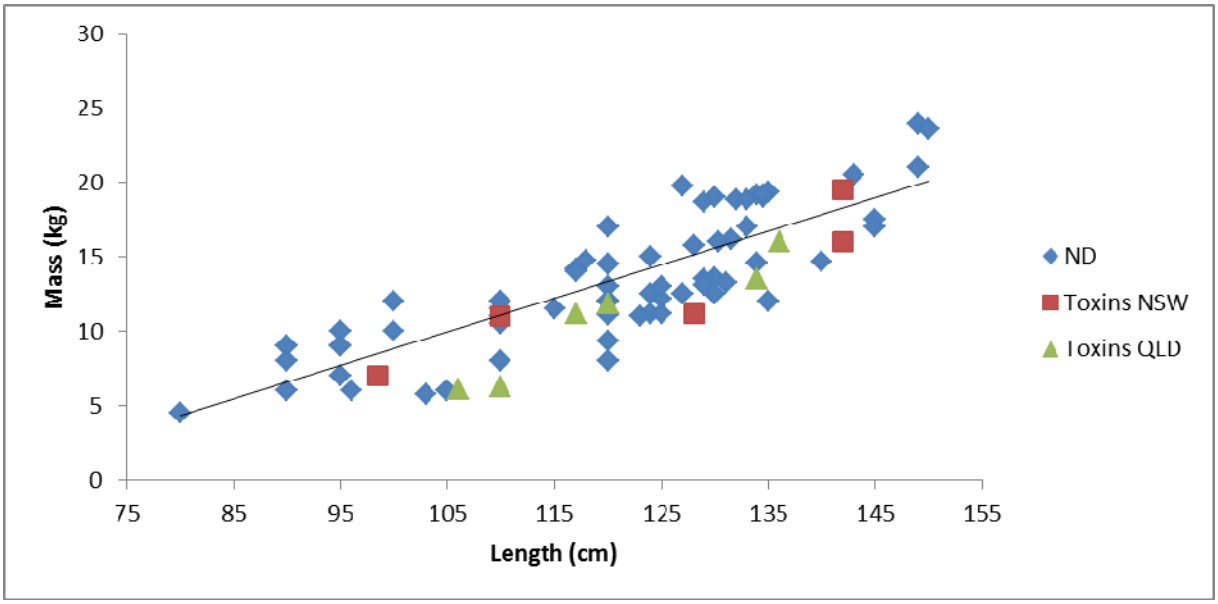


Figure 14: Relationship between the weight and length of toxic/non-toxic fish caught in NSW, QLD and previous 2014-2015 CFP incidents in NSW (n=87). (ND- fish specimen in which P-CTX-1B levels were not detected ; Toxins NSW- fish specimen in which P-CTX-1B levels were detected in fish caught in NSW, Toxins Qld – fish specimens in which P-CTX-1B levels were detected in fish caught in QLD).

8.4 Species identification

We confirmed the identity of every specimen as *S. commerson*, as all fish liver samples showed amplification with the qPCR assay, specific for *S. commerson*, described in section 8.3.

9 Discussion

The first objective of this project was to set up the first facility with the capability of measuring P-CTX-1B in samples from NSW. The results show that the facility at the Sydney Institute of Marine Science in Sydney successfully developed and implemented this capability due to the significant input from the Cawthron Institute, New Zealand, who operate the Safe New Zealand Seafood research programme, which includes ciguatera research as a priority. In August 2015, the facility at SIMs was used to determine whether fish from the Capel Banks region in the Coral Sea, which had not previously been regulated or known for CTX presence, contained detectable levels of P-CTX-1B. These samples were found to have detectable levels of P-CTX-1B toxins (Table 10), and effective management related decisions could be made within a few days of the first sample provision. This demonstrates that the objective of setting up a viable facility that can measure P-CTX-1B in Sydney, on industry-relevant samples, on an as-needed basis and with a rapid turnaround time, has been achieved.

The capability to continue detecting P-CTX-1B in samples using this facility is entirely dependent on whether it is possible to source further CTX toxin standards. These are needed as a comparison to confirm the identity of toxins that are present. The LC-MS facilities at SIMs (and at Cawthron) have very limited supplies of P-CTX1B, obtained through collaborations with individual researchers in government and university laboratories internationally. The supplies of the SIMs facility were largely exhausted for this study alone. A difficulty in sourcing toxin standards is a problem not confined to this facility, but is known internationally, and is discussed at length in the UNESCO IOC Global Ciguatera Strategy 2015- 2019 (IOC-UNESCO, 2015). If access to a facility for testing for CTX presence is required into the future, there is an urgent need to develop more standards and/or determine proxy methods for CTX analyses.

The second objective of this study was to show the first evidence that P-CTX-1B toxins are present in a random sample of Spanish Mackerel from NSW waters. The results indicate that these toxins do occur in NSW, in fish not previously known to be associated with any CFP illnesses. Previously, the only Spanish Mackerel from NSW that had been tested and found to carry CTX toxins were those few individual fish that were sampled after the event, as the remains of a meal, due to their presumptive role in CFP illnesses (Farrell *et al* 2016).

In Spanish Mackerel randomly sampled from NSW waters in this study, as opposed to those analysed from NSW waters following CFP illnesses, 1 in 71 fish were positive for P-CTX-1B in the flesh samples, which would indicate a 1.4% prevalence (1% - 4%, as lower and upper values, based on 95% confidence intervals) at the sampled sites. In the liver samples, 5 in 71 fish were positive for P-CTX-1B, which would indicate a 7% prevalence (1% - 12%, as lower and upper values, based on 95% confidence intervals) at the sampled sites. We consider this to represent the Spanish Mackerel fishery in NSW relatively well, as we covered the vast majority of the region from which Spanish Mackerel are caught in NSW, with the majority from the Coffs Harbour region, and also captured relatively accurately the percentage caught by the recreational community as compared to the commercial fishing community.

We also examined a small number of samples from QLD (n=13), of which 6 were found to be positive in the flesh for P-CTX-1B (an incidence rate of 46%, but 95% confidence intervals of 19-73%). As examining the rate of CTX in Spanish Mackerel in QLD was not the aim of this study, these data are considered only exploratory, and they accordingly represent a very small sample size.

These incident rates need to be taken as only indicative rather than final, as there are several caveats associated with them. Firstly, any final percentage prevalence rate is subject to relatively high confidence intervals, as discussed above. Secondly, this study was limited to samples that were returned to us (84 of 400 sample packs that were distributed) from the recreational and commercial fishing community. While the sites at which samples were taken broadly represents the major locations

(Coffs Harbour, Byron Bay) and the relative distribution of commercial compared to recreational fisheries catches of Spanish Mackerel in NSW (~1:10 ratio), it was not designed to emulate the distribution of the total NSW catch in a detailed way. A larger study with a sampling framework designed to emulate the distribution of the catch in NSW needs to be undertaken in order to gain an accurate estimate of the prevalence rates.

In the analysis of the length/weight of the fish that were found to have significant levels of CTX-1B, no relationship could be seen between these two variables. This is similar to what has been found in the study on CTX levels in Barracuda in the Caribbean (Dechraoui *et al.*, 2005), in which no correlation was found between CTX levels in liver and fish size. A recent study which analysed CTX levels in fish using the receptor binding assay from 45 species in French Polynesia, including a total of 856 individual fish, also found that there was a positive correlation between fish size and CTX levels in only one species (*Lutjanus bohar*, Red Bass) of the 45 species assessed (Gaboriau *et al* 2014) The others showed no clear relationship, except for two fish species, which showed a negative relationship (smaller fish had higher toxin levels). They concluded that fish size cannot be used as a universal predictor of likely fish CTX levels in French Polynesia, and that more research needs to be undertaken into the processes of CTX bioaccumulation and depuration in individual fish species (Gaboriau *et al* 2014). In contrast, as discussed in Section 5.4 of the introduction, a clear positive relationship between fish size and CTX levels was found for four fish species in Japan (Oshiro *et al* 2010), one of which was *L. bohar*. These data indicate that a relationship between fish size and CTX levels may differ on a species-specific, and/or a regional basis, and therefore likely needs to be verified for individual fisheries.

There are further uncertainties in these data, due to factors that are beyond the scope of this study. We contacted all those who supplied fish that we detected as positive for P-CTX 1B, to further question them regarding any possible illness reports. While not all those contacted responded, and it is not possible to ascertain whether the CTX positive fish in this study were consumed, no CFP-like illnesses have been reported to date due to fish from the random study. This indicates that there is a need to further understand the relationship between the levels of P-CTX-1B in Australia in relation to CFP illnesses. The levels of P-CTX-1B in fish that are correlated with CFP illnesses has been found to vary (see discussion below), due to many differing factors. Each of the individual Spanish Mackerel that we have identified as positive for P-CTX-1B in this study had the potential to cause illness, as their levels of P-CTX-1B were greater than or approximately 0.1 $\mu g\ kg^{-1}$, which is 10 times the US FDA "guidance level" and at a similar level to that found in fish flesh known to have caused illness previously (Tables 10, 11).

Table 10: P-CTX-1B levels in fish known to be associated with illness with CFP symptoms in Australia.

Location	Fish species	P-CTX-1B in flesh ($\mu g\ kg^{-1}$)	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	06-1.0	(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 11: Toxicity and level of P-CTX1B in leftover meals from CFP incidents in Japan (Oshiro *et al.*, 2010). 1 MU toxicity equals 7 ng of P-CTX-1B in fish flesh (Yasumoto, 2005).

CFP number 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.

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 CFP illnesses in Australia (Table 10) and overseas (

Table 11~~Table 11~~). This shows that levels above ~0.1 µg kg-1 have been known to be associated with illness~~es~~, 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 10 and 11). 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 LCMS, 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.

10 Conclusion

1. We have succeeded in setting up a viable commercial testing facility for P-CTX-1B in NSW. This facility has already been used to determine, within 2 days, whether fish from a relatively little known fishing region, Capel Banks in the Coral Sea, not previously regulated or known for CTX presence, contained detectable levels of P-CTX-1B.

2. There is an urgent need for access to CTX standards in order that analyses such as these can continue. The current study focused on a single CTX analogue, the fish metabolite known as ciguatoxin-1B (P-CTX-1B), as it is a common analog found in ciguatoxic fish found in the Australian region. However, several other potent analogues exist in fish in this region. In addition, the LC-MS facility at SIMs, in line with other research groups, have very limited supplies of P-CTX-1B, obtained largely through collaborations with individual researchers in government and university laboratories internationally. These supplies were largely exhausted for this study alone. Therefore, if access to a facility for testing is required into the future, there is an urgent need to conduct a study to develop more standards and/or determine proxy methods for CTX standards.

3. In NSW waters, , 1 in 71 fish caught as part of our sampling study, as compared to fish we analysed from known CFP cases, were positive for P-CTX-1B in the flesh samples, which would indicate a 1.4% prevalence (1% - 4%, as lower and upper values, based on 95% confidence intervals) at the sampled sites. In the liver samples, 5 in 71 fish were positive for P-CTX-1B, which would indicate a 7% prevalence (1% - 12%, as lower and upper values, based on 95% confidence intervals) at the sampled sites. However, several caveats exist in relation to these abundance estimates. Firstly, the sample sizes were relatively low. Secondly, although fish were caught from the main regions that supply the recreational and commercial fishing community, samples were opportunistic and not designed to exactly mimic the total catch from NSW. Thirdly, other CTX analogs exist that were not monitored for, therefore total toxicity may vary among fish.

4. The levels of P-CTX-1B that lead to illness in Australia may require more investigation, in order to determine whether the US FDA guidance level is appropriate for Australia.

5. In the analysis of the length/weight of the fish that were found to have significant levels of CTX-1B in this study, no relationship could be seen between these two variables in Spanish Mackerel.

11 Recommendations

1. In order to set appropriate risk management procedures, we require an increased understanding of the levels of ciguatoxins, including CTX-1B, in Australian fish samples and the likelihood of these causing human illness. The FDA in the US has set a low 0.01 ppb 'P-CTX-1B equivalents' guidance limit in their specifications. At present, this low level is extremely difficult to detect using existing LC-MS technology. The use of total ciguatoxicity assays (including functional assays such as the neuroblastoma bioassay, or the CTX receptor binding assay, and/or others) in comparison to LC-MS would enable a more detailed risk evaluation. There is a need to determine if this US FDA specification is set at an appropriate level with regard to ciguatera risk in Australian Spanish Mackerel, as well as other Australian fish that are known to potentially contain CTX.

2. The current study was limited to the opportunistic collection of samples from NSW (n=71) and a very small subsample from QLD (n=13), because the study was concerned with fish from NSW. A more broadscale overview of the levels of ciguatoxin in Spanish Mackerel (liver and flesh) and the potential risk to consumers would be determined by expanding the geographic range and sample size in a national baseline survey.

3. The industry and recreational sector will need to determine the cost/benefit ratio regarding whether these data can be built on, so that samples can be collected and analysed to allow for a thorough, scientifically robust, food safety risk evaluation to be undertaken on the risk of consuming Spanish Mackerel caught in Australian, not only NSW, waters.

4. To carry out any further analysis of CTX in fish in Australia, there will be a need to obtain further ciguatoxin reference material to act as standards for the testing process.

12 References

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