Determination of the Presence and Biomagnification of Caribbean ciguatoxins and benthic algal toxins in fishes from the Florida Keys National Marine Sanctuary

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The final copy of this thesis has been examined by the signatories, and we find that both the content and the form meet acceptable presentation standards of scholarly work in the above mentioned discipline.

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Abstract

Ciguatera fish poisoning (CFP) is a common syndrome affecting coastal communities in the Atlantic and Indo-Pacific regions, including the Greater Caribbean. This severe illness is caused by the ingestion of reef fish contaminated with ciguatoxins (CTXs). The source of CTXs has been associated with epiphytic dinoflagellates of the genus *Gambierdiscus* (among others) which reside on macroalgae that are grazed by herbivores. The entry of algal CTX precursors (often referred to as gambiertoxins) into the food web initiates a cascade of trophic transfer events and biotransformations that have not yet been fully elucidated in the Greater Caribbean region. A critical knowledge gap addressed in the present study was to gain better predictive capability of CTX presence in fishes across trophic levels in a sub-tropical hotspot for CFP (i.e. the Florida Keys). A variety of fish including herbivores (e.g., representatives of Acanthuridae and Pomacathidae) up to apex predators (e.g., *Sphyrena barracuda* and *Mycteroperca bonaci*) were collected from a long-term monitoring site in the Florida Keys. Extracts of fish were assessed for neurotoxicity and CTX activity using the sensitive *in vitro* neuroblastoma (N2a) assay. Twenty five of the 66 fish (38%) examined in this study expressed sodium channel-dependent toxicity in both sensitized and non-sensitized cell treatments, highlighting the likely presence of multiple toxin classes in these reef fish. Monospecific sodium channel activity was observed in 3% of fish represented by one species: *S. barracuda.* Stable isotope analysis was used to determine fish trophic level and in the calculation of a food web magnification factor (FWMF) of 1.114 for CTX and CTX-like compounds, indicative of the biomagnification of these toxins. The presence of toxic fish indicates a need for risk assessment and management consideration in the Florida Keys.

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Introduction

Ciguatera fish poisoning (CFP) is a worldwide health epidemic affecting human populations in tropical and subtropical zones that is caused by the ingestion of seafood (primarily fish) that contain naturally-occurring toxins produced in the environment (de Sylva, 1994; Lewis, 2001; Bienfang et al., 2011; Richlen et al., 2012; Ledreux et al., 2014; Rains & Parsons, 2015). The earliest records of this epidemic can be traced back to the 16th century in writings by Pedro Martyr de Anghera in the islands of the Caribbean Sea (MacNutt, 1912). The first report from the Pacific Ocean came from the Portuguese explorer Pedro Fernandez de Queiros, who reported in 1606, that sailors ate fish from the island currently known as Vanuatu and became ill (Halsted, 1967). These historical reports suggest that toxic fish have existed long before Europeans traveled to the tropics. Human population expansion and the development of a globally connected tropical fish market, however, have increased fish consumption and could possibly increase CFP outbreaks in the future.

There are 50,000 cases of CFP reported per year; however, there are numerous cases that are misdiagnosed as neurotoxic shellfish poisoning (NSP) or paralytic shellfish poisoning (PSP), or that are not reported at all (Manger et al., 1995). Adjusting for misdiagnoses and underreporting, an estimated 500,000 cases of CFP are thought to occur per year (Bienfang et al., 2011). According to de Sylva (1994), four counties in the state of Florida (Dade, Broward, Monroe, and Palm Beach), account for 1,300 CFP cases per year. Additionally, in the Caribbean region, CFP causes yearly economic losses on the order of $10 million from medical costs and negative publicity, which have led to liability insurance cases (Anderson et al., 2000). Another study found an average

negative economic impact of $21 million per year for endemic regions in the continental United States (Anderson et al., 2000). Furthermore, this value is variable because of the ratio of reported to underreported cases of CFP: 1:4 in Florida, 1:10 in the Northern Mariana Islands and American Samoa, and 1:100 in Hawaii, Guam, Puerto Rico, and the U.S. Virgin Islands (Anderson et al., 2000). The most recent study in Florida found the incidence rate of CFP to be 5.6 per 100,000 Floridians, equivalent to an estimated 1,000 people per year (Radke et al., 2015). In Florida and the Greater Caribbean region, there are little to no data on changes in CFP cases over time, though the incidence rate is very high in certain areas of the Caribbean. Only one known study has examined long-term trends of CFP, suggesting that the incidence is significantly higher based upon data collected, but there is still uncertainty surrounding temporal trends of this disease (Radke et al., 2015).

Early investigations into CFP outbreaks throughout the Pacific Islands found that the geographic distribution of toxic fish species is inconsistent, hypothesized to be due to variations of fish diets on coral reefs (Dawson et al., 1955). Randall (1958) completed an extensive review of ciguatera around the world and made multiple postulations: (1) fish toxicity has some association with fish diet, (2) the toxic organism is most likely an algae, fungus, protozoan, or bacteria that accidentally gets consumed by primary consumers, and (3) most toxic fish are large predatory species that feed on other fish species, allowing for toxin assimilation and storage. Cooper (1964) did a large-scale survey of CFP cases in the Gilbert Islands attempting to understand how these intoxications varied from island to island as well as the cyclic evolution of toxic conditions over multiple years from different reefs in the island chain. Studies that

followed produced results that supported Randall's postulations and set the stage for future studies, which found CFP to be caused by a suite of strongly polar, heat-stable, lipid-soluble, highly oxygenated, cyclic polyether molecules called ciguatoxins (CTX) originating from the less polar molecules, gambiertoxins (GTX) (Helfrich and Banner, 1963; Banner et al., 1966; Scheuer et al., 1969; Lewis, 2001; Rains and Parsons, 2015). These toxins (CTX-1 and GTX-4B) were first characterized by Murata et al. (1990), from extracted *Gymnothorax javanicus* (the giant moray eel) viscera and biodetritus from the Pacific Ocean using nuclear magnetic resonance spectroscopy (NMR) and mass spectral measurements. Continual characterization and purification of moray eel viscera revealed two new CTXs, CTX-2 and CTX-3, which are related chemically to previously identified CTXs (Lewis et al., 1991). GTXs are produced by benthic epiphytic dinoflagellates of the genus *Gambierdiscus*, which was first implicated as a source of toxin by Yasumoto et al. (1977a). The dinoflagellate was later described as *Gambierdiscus toxicus* by Adachi and Fukuyo (1979) in recognition of the discovery of this dinoflagellate in the Gambier Islands, French Polynesia. The epiphytic nature of *Gambierdiscus* sp. allows herbivorous fish and invertebrates to consume these cells when grazing upon macroalgae, initiating food chain transfer of GTX and CTX (Randall, 1958; Helfrich and Banner, 1963; Banner et al., 1966; Banner, 1974; Lewis & Holmes, 1993; Rains and Parsons, 2015). The presence of *Gambierdiscus* sp. in the gut contents of *Naso unicornis* (unicornfish) from areas that are chronically toxic and absent in the gut contents of fish from less toxic areas supported the theory of food chain transfer of CTX in reef environments (Yasumoto et al., 1977b). Understanding the factors that influence the presence and abundance of *Gambierdiscus* cells on a reef is the first step to understand the distribution of CTXs

throughout the food web (Legrand 1998; Parsons et al., 2010; Parsons et al., 2011; Rains and Parsons, 2015; Parsons et al., 2017). *Gambierdiscus* sp. populations on host macroalgae at specific reefs would give a baseline of possible toxin influx based upon dominating "ciguatoxin-super-producing" species (Legrand 1998). Several studies on toxin production by specific strains from tropical, subtropical, and temperate regions have revealed certain strains that are more toxic than others (Chinain et al., 2010; Lewis et al., 2016; Litaker et al., 2017; Pisapia et al., 2017). There is a consensus that *Gambierdiscus polynesiensis* is likely the super-producing ciguatoxic species for the Pacific region; however, whether *G. silvae* or *G. excentricus* is the super-producing ciguatoxic species for the Caribbean region is still up for debate (Chinain et al., 2010; Lewis et al., 2016; Litaker et al., 2017). Understanding *Gambierdiscus* sp. dynamics on host macroalgae on coral reefs is an integral step in creating a future food safety management plan.

Over 400 species of fishes have been implicated in CFP cases around the world, most notably game fish such as grouper (*Serranidae*), barracuda (*Sphyraenidae*), snapper (*Lutjanidae*), jacks (*Carangidae*), and mackerel (*Scombrini*) (Cooper, 1964; Halstead, 1967; Lange, 1994; Robertson et al., 2014). CTXs are not influenced by cooking or freezing, and a ciguatoxic fish is not identifiable from any visual cues, such as color, odor, or taste (Bienfang et al., 2011; Robertson et al., 2014). When CTXs are consumed by humans, perturbations occur along the voltage-gated sodium channels (VGSC) where CTX specifically binds to receptor site 5. This binding creates a negatively charged shift along the channel membrane, blocking inactivation, and results in an influx of sodium ions (Na^+) into the neuron (Huang et al., 1984; Benoit et al., 1986; Lombet et al., 1987;

Manger et al., 1995; Cestele & Catterall, 2000). This influx of Na^{+} into the cell directly increases internal calcium ions (Ca^{+2}) via the Na^{+} - Ca^{+2} exchange, which then leads to the swelling of neurons with water to compensate for intracellular Na^{+} and Ca^{+2} induced by CTX (Lewis & Endean, 1986; Seino et al., 1988; Mologo et al., 1992). CFP symptoms commonly arise within the first 12 hours of toxic fish consumption; however, with the consumption of extremely toxic fish, symptoms can arise within 30 minutes, causing gastrointestinal, neurological, cardiovascular disturbances and, in extreme cases, paralysis, coma, or death (Bagnis et al., 1979; Lewis & Holmes, 1993; Hirama et al., 2001; Robertson et al., 2014). Specific symptoms, such as lethargy, itching of the skin, reversal of temperature sensations, tingling and numbness of the lips, hands and/or feet, weakness and pain in joints and muscles can last from a few days to weeks, or possibly up to months, depending on dose and individual vulnerability (Cooper, 1964; Bagnis et al., 1979; Bagnis & Legrand, 1988; Lewis & Holmes, 1993; Lewis, 2001). There are currently no treatments for CFP, although there has been variable success in reducing the severity of symptoms using mannitol within the first 24 hours of onset, but symptoms may reoccur after treatment ceases (Palafox et al., 1988). There are documented successful treatments in the Pacific and Caribbean regions with full recovery after two to three days; conversely, these cases are few and are dependent upon the individual's susceptibility to intoxication (Morris et al., 1982; Palafox et al., 1988; Stewart 1991; Blythe et al., 1992; Lange et al., 1992; Eastaugh, 1996). Creating a recognized and standardized treatment that the medical community approves for future outbreaks would be a positive first step to mitigate intoxication of global populations.

A total of 29 congeners of CTX have been identified to date with CTX-4A, CTX-4B, CTX-3, CTX-2, and CTX-1 as the most commonly discussed congeners, and each interacts differently with voltage gated sodium channels (VGSCs) because of their specific chemical structures and properties (Murata et al., 1989; Manger et al., 1990; Murata et al., 1990; Lewis et al., 1991; Lewis and Holmes, 1993; Hamilton et al., 2002). CTX-4A and CTX-4B are the least polar and stable molecules out of the CTXs and are produced directly by *Gambierdiscus*, which is subsequently grazed upon by herbivores, initiating the oxidative and metabolized processes by fish through the food web (Lewis and Holmes, 1993). CTX-4A and CTX-4B are differentiated by their "back-bones", as observed when purifying and characterizing these toxins from different *Gambierdiscus* sp. and from different organs in fish that metabolized these congeners (Lewis and Holmes, 1993; Lehane and Lewis, 2000). CTX-4A analogs gives rise to CTX-2 while CTX-4B analogs gives rise to CTX-3 and then CTX-1 when oxidized by fish metabolic processes (Lewis and Holmes, 1993). CTX-2 and CTX-3 are generally more abundant in lower trophic level fish species, and are categorized as less stable, less polar, and less potent toxins compared to CTX-1, due to their being less oxygenated molecules with fewer hydroxyl groups present (Lewis and Holmes, 1993). CTX-1 is thought to be the major toxin causing CFP, and is commonly found in predatory and higher trophic level species that are readily available for human consumption. This congener is stable and well oxygenated with hydroxyl groups making the molecule highly polar for a lipophilic compound (Manger et al., 1990; Lewis & Holmes, 1993; Lewis 2001). The elimination and depuration rates for specific CTX congeners in fish are still unknown; however, there have been a few studies attempted to determine general CTX depuration rates in fish.

Banner et al. (1966) were able to determine that CTXs in wild caught red snapper (*Lutjanus bohar*), a common fish species to cause CFP in the Pacific Ocean, were able to retain relatively moderate toxicity over a 30-month period while being fed non-toxic diets in large aquaria and ponds. Lewis et al. (1992) were able to determine a half-life of 264 days for CTX from the analysis of 217 wild caught moray eel (*Muraenidae*) viscera. Additionally, they found that the major component (90%) of the toxin present was an extremely polar compound (for a lipophilic molecule), most likely CTX-1, and that the minor component (10%) was composed of less polar compounds (uncharacterized). The presence of multiple CTXs (-1, -2, and -3) in fish implicates the importance of ciguatera management since these congeners can alter illness occurrence, diagnosis, and future treatments (Lewis and Sellin, 1992; Lewis and Jones, 1997).

The toxicokinetics of how and where CTXs are being transported through the body of an individual organism are beginning to be better understood for fish and mammals from recent laboratory studies in mullet (*Mugil cephalus*) and rats in particular (Bottein Dechraoui et al., 2011; Ledreux and Ramsdell, 2013; Ledreux et al., 2014). The initial absorption and bioavailability of CTXs has been shown through intraperitoneal (ip) and oral injections, to be 75%, and 39% efficient, respectively, in blood circulation for rats (Bottein Dechraoui et al., 2011; Ledreux and Ramsdell, 2013). The absorption of CTXs into the blood of mullet that were fed *Gambierdiscus polynesiensis* cells was 42%, which is notably similar to the rat absorption (Bottein Dechraoui et al., 2011; Ledreux and Ramsdell, 2013; Ledreux et al., 2014). The circulation and storage of CTXs throughout the body appears to be rapid in rats. For example, the highest organ burden and accumulation of CTXs after 96-hour ip and oral exposures were muscle > brain > liver

and muscle > liver > brain, respectively (Dechraoui Bottein et al., 2011). For mullet, the site of highest storage of CTXs after 24 hours from a single feeding of *G. polynesiensis* was the intestines and the muscle; however, only 5% of the absorbed CTXs were retained and found in the tissues of these fish (Ledreux et al., 2014). Ledreux et al. (2014) also conducted an experiment doing repeated exposures of CTXs in mullet (1, 2, 6, and 9 exposures) that indicated that CTX concentrations do not increase with multiple exposures and that there is a possible saturation limit, which has also been observed for short-nosed unicornfish (*Naso brevirostris*) (Clausing et al., 2016). The terminal half-life of CTXs in blood from rats and mullet was estimated to be 4 days and 4.2 hours, respectively, and the blood clearance rate for rats of 0.69 mL/h/g for intravenous (iv) exposures, which was found to be close to the same rate as oral and ip exposures (Bottein Dechraoui et al., 2011; Ledreux and Ramsdell, 2013; Ledreux et al., 2014). Similar elimination rates of CTXs from blood in rats through iv, ip, or oral exposure, indicates that there appears to be no difference in the route of exposure (Ledreux and Ramdsell, 2013). Bottein Dechraoui et al. (2013) were also able to determine the excretion rates from rats in urine and feces over the 96-hour ip and oral exposures of CTX which indicated significantly higher elimination rates through feces than through urine. Similar findings were found by Ledreux et al. (2014) for mullet, who reported high concentrations of CTXs are eventually eliminated by the intestines and gall bladder through biliary routes. Such studies as these are important, as understanding the toxicokinetics of a surrogate fish and mammal organism can help determine the movement of CTXs when consumed by humans.

As fish consume lipophilic compounds, like CTXs, metabolic processes initiate in the stomach, liver, and kidneys that result in a suite of toxins that increase potency than compared to the parent compound, like processes associated with ingestion of hydrocarbons (Gingerich, 1982; Lech et al., 1982; Lewis and Holmes, 1993). CTXs undergo biotransformation, chemical metabolic reactions mediated and expedited by enzymes within the fish to convert parent chemicals into polar metabolites (Buhler and Williams, 1988). Biotransformation and metabolic pathways are used for a multitude of different lipophilic organic toxins, such as most pharmaceuticals, steroid hormones, bile acids, fatty acids and prostaglandins, to increase the polarity and hydrophilic properties of these toxins to be more readily eliminated (Zeldin and Seubert, 2008; Celander, 2011). Phase I of biotransformation is the oxidation reaction catalyzed by the cytochrome P-450 (Cyps) monooxygenase systems (such as oxidases, reductases, and dehydrogenases) resulting in a more polar metabolite to facilitate possible excretion (Buhler and Williams, 1988; Goksøyr and Förlin, 1992; Ziegler, 1994). Phase II of this process is conjugation, which is the attachment of endogenous molecules by enzymatic reactions (such as glucuronidation, sulfation, methylation, N-acetylation), and attachment of glutathione or amino acids to create an even more polar metabolite (Handschin and Meyer, 2003; Celander, 2011). Finally, phase III reactions occur which in transporter proteins and efflux activities process sends metabolized CTXs to be slowly eliminated or stored in the body of the fish. The result of these metabolic reactions and pathways is the causative toxin of CFP and the most abundant toxin in higher trophic level fish species, CTX-1 (Murata et al., 1990; Holmes et al., 1991; Lewis & Holmes, 1993; Lehane and Lewis, 2000; Yasumoto et al., 2000; Mak et al., 2013; Yogi et al., 2014; Meyer et al., 2016;

Diogène et al., 2017). Phase I biotransformation pathways have been shown for CTXs using both human and fish Cyps in the laboratory using CTX standards (Ikehara et al., 2017). Phase I and II reactions were also documented for ladder-like polyether toxins like CTXs (brevetoxins), both of which adhere to site 5 on VGSC, based on liver genomic responses in mice, rats, and striped bass (*Morone saxatilis*) using standard toxic material (Washburn et al., 1996; Walsh et al., 2003; Radwan and Ramsdell, 2006; Morey et al., 2008). As fish biotransform CTXs, they continue to consume these toxins, resulting in a compounding bioaccumulation effect due to the slow elimination and depuration of this suite of toxins (Banner, 1966). CTX biotransformation and bioaccumulation can lead to higher toxin concentrations in fish tissue that are then directly consumed by humans.

Geographic regions play an integral role for CFP management, symptomology, and care for affected individuals. CTXs are grouped into three specific regions: Pacific CTX (P-CTX), Caribbean CTX (C-CTX), and Indian Ocean CTX (I-CTX) (Holt et al., 1984; Murata et al., 1989; Murata et al., 1990; Dickey et al., 1995; Crouch et al., 1995; Hamilton et al., 2002). CTX lethal doses (LD_{50}) for P-CTX-1 and C-CTX-1, are 0.25 and 3.7 ug CTX/kg body weight ppm when injected into mice respectively; there is currently no LD_{50} for I-CTX (Abraham et al., 2012; Meyer et al., 2016). P-CTX-1 is the most oxidized form and is thought to be ten times more potent compared to all other forms of CTX. P-CTX-1 has been closely studied in the Pacific as it is the prevailing toxin found in carnivorous fish species (Murata et al., 1989; Murata et al., 1990; Lewis et al., 1991; Lewis & Holmes, 1993; Lewis, 2001). Based on 3,009 cases of CFP in the south Pacific, it is suggested that P-CTXs cause more neurological symptoms such as paresthesia of extremities, circum oral paresthesia, burning or pain to skin on contact with cold water,

arthralgia, and myalgia (Bagnis et al., 1979; Bagnis & Legrand, 1988; Lewis et al., 1988; Ferner et al., 1997). On the other hand, while C-CTX and I-CTX are highly oxidized, they are thought to be less potent and very little is known about the ability of these toxins to move throughout the food web or their resultant human health effects (Lewis 2001; Abraham et al., 2012). From the limited epidemiological studies conducted, I-CTX causes symptoms of paresthesia, asthenia, dysesthesia, nausea, vomiting, diarrhea, and strangely hallucinatory effects (Habermehl et al., 1994; Quod & Turquet, 1996). C-CTX was first purified in extremely small quantities by Dickey et al. (1995) and Crouch et al. (1995). It was then structurally configured by Lewis et al. (1998), using great barracuda (*Sphyraena barracuda*) and horse-eye jack (*Caranx latus*) toxic extracts. Epidemiological case studies agree that the most common symptoms associated with C-CTX are gastrointestinal and digestive discomfort (30% to 80% of all reported Caribbean cases), which can cause serious dehydration resulting in hypovolemia and shock (Lawrence et al., 1980; Holt et al., 1984; Poli et al., 1997; Pottier et al., 2002). C-CTX can also cause cardiovascular symptoms (reported symptom in >50% of Caribbean CFP cases) such as hypotension and bradycardia (<60 beats min^{-1}), which can be extremely serious and could lead to mortality if not treated quickly (Hanno, 1981; Geller & Benowitz, 1992; Pottier et al., 2001). Understanding the symptomology associated with geographic differences may be a successful way to assess human population risk of contracting CFP.

Understanding the capability of CTX and CTX-like compounds to biomagnify in coral reef ecosystems is an extremely difficult objective. These toxins biotransform and bioaccumulate at different rates within fish, causing the formation of multiple toxin metabolites, which can result in different biological responses, complicating the risk

assessment of reef fish susceptibility to toxin uptake and transference (Chungue et al., 1976; Bagnis et al., 1980; Pottier et al., 2002; Llewellyn, 2010). Trophic transfer of toxins is an important factor for understanding toxin fluctuations in reef food webs (Lewis et al., 1988; Vernoux et al., 1985; Oshiro et al., 2010; Clua et al., 2011). To date, three studies have looked into the trophic transfer of CTX and CTX-like compounds in reef fish; however, these have only targeted the Pacific region, leaving a gap of knowledge for the Caribbean endemic region (Chan, et al., 2011; Mak et al., 2013; Gaboriau et al., 2014). The main vector for toxin entering the reef food web in the Pacific has been identified to be herbivorous fish such as surgeonfish (*Acanthuridae*) and parrotfish (*Scaridae*), which graze upon macroalgae and turf algae, thus ingesting toxic epiphytic dinoflagellates (Mak et al., 2013). Conversely, Caribbean region herbivores, such as surgeonfish (*Acanthuridae*) and parrotfish (*Scaridae*), have been regularly consumed in the past and have not caused CFP symptoms indicating that the toxin entry point in Caribbean reef food webs may be more convoluted (Czernichow et al., 1984).

This study will be the first to target CTX and CTX-like compounds that bioaccumulate and biomagnify across trophic levels in the Caribbean region, specifically in the Florida Keys. Specifically, the goals of this study are to: (1) determine the presence of CTX and CTX-like compounds in coral reef fish in the Florida Keys National Marine Sanctuary (FKNMS), (2) identify the introductory vector of these toxins into the food web; and (3) quantify CTX and CTX-like compounds to determine if these toxins are biomagnifying.

Methods

Field Sampling/Initial Processing

Fish species were collected in the year of 2013 from Tennessee Reef Lighthouse (Figure 1) off of Long Key, Florida (maximum depth range from 6 to 8 m) by SCUBA spearfishing and rod and reel methods. Fish species that were collected from this site and the sample size for each can be found in Table 1. Whole fish were put in bags and labeled with site and month collected. Bagged fish were placed in a - 20˚C freezer prior to processing. Processing included assigning each fish an identification number along with total length (mm), forked length (mm), and whole fish mass (g) measurements, as well as taxonomic classification down to the genus and species level. Five gram (g) subsamples of fillet were haphazardly chosen, weighed out, placed in labeled glass scintillation vials and stored at - 20˚C. All remaining fillet tissue was weighed and placed in Ziploc bags labeled with the fish identification number, site, month and year collected, and mass of remaining fillet tissue and placed in a -20˚C freezer for further toxin analysis.

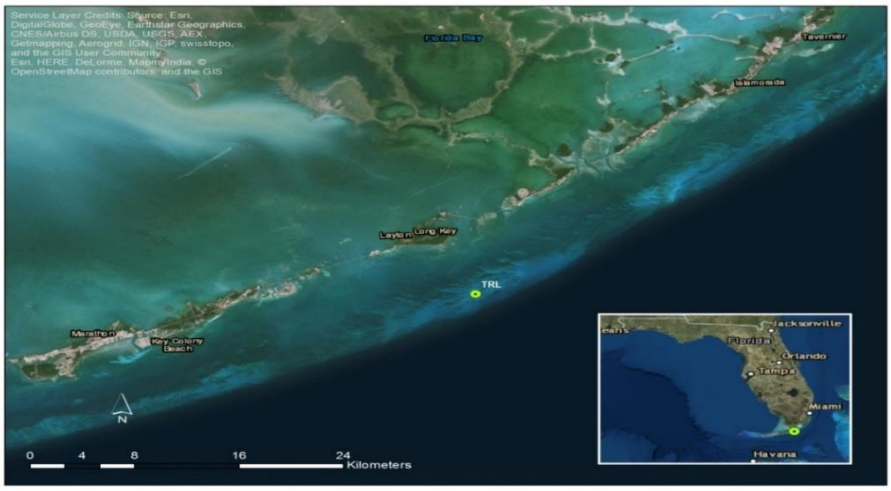


Figure 1: Map of Florida Keys with Tennessee Reef Lighthouse marked as TRL.

Table 1: List of fish species collected from TRL in the Florida Keys.

Family	**Genus**	**Species**	**Common Name**	***n***
Acanthuridae	*Acanthurus*	*coeruleus*	Blue Tang	13
Monacanthidae	*Aluterus*	*scriptus*	Scrawled Filefish	2
Haemulidae	*Anisotremus*	*virginicus*	Porkfish	9
Labridae	*Bodianus*	*rufus*	Spanish Hogfish	1
Sparidae	*Calamus*	*calamus*	Saucereye Porgy	1
Sparidae	*Calamus*	*nodosus*	Knobbed Porgy	1
Carangidae	*Caranx*	*crysos*	Blue Runner Jack	2
Serranidae	*Cephalopholis*	*cruentata*	Graysby Grouper	1
Serranidae	*Epinephelus*	*adscensionis*	Rock Hind Grouper	1
Pomacanthidae	*Holacanthus*	*tricolor*	Rock Beauty Angelfish	1
Kyphosidae	*Kyphosus*	*sectatrix*	Bermuda Chub	3
Labridae	*Lachnolaimus*	*maximus*	Hogfish	7
Lutjanidae	*Lutjanus*	*apodus*	Schoolmaster Snapper	1
Serranidae	*Mycteroperca*	*bonaci*	Black Grouper	3
Pomacanthidae	*Pomacanthus*	*arcuatus*	Gray Angelfish	3
Pomacanthidae	*Pomacanthus*	*paru*	French Angelfish	1
Mullidae	*Pseudupeneus*	*maculatus*	Spotted Goatfish	3
Scaridae	*Sparisoma*	*aurofrenatum*	Redband Parrotfish	1
Sphyraena	*Sphyraena*	*barracuda*	Great Barracuda	11
Pomacentridae	*Stegastes*	*partitus*	Bicolor Damselfish	1
			Total	**66**

Toxin Extraction

Fish tissue were removed from the freezer and thawed. After thawing, fish tissue was initially extracted twice by grinding using a Bead Rupter 12 (OMNI International the Homogenizer Company^{TM}, Kennesaw, Georgia) with HPLC grade acetone (2 mL/g).

Acetone extracts were pooled together for each fish and placed in a - 20˚C freezer for at least 24 hours to precipitate out unwanted proteins, which were later discarded. Pooled extracts were centrifuged, then supernatants containing possible CTX or CTX-like compounds were removed and dried under a steady stream of industrial grade nitrogen. Dried residue was reconstituted three times in HPLC grade 90% aqueous methanol (1 mL/g) and HPLC grade *n*-hexanes (2 mL/g) to remove non-polar lipids. Extract was then centrifuged to effectively separate reagents with the top hexane portion being removed as waste. The leftover aqueous methanol solution was dried down again under a stream of nitrogen. Dried residue was reconstituted twice in HPLC grade water (1 mL/g) and HPLC grade chloroform (1 mL/g) to collect the polar lipids, which contain CTX and CTX-like compounds. Extract was centrifuged each time to effectively separate reagents, and the lower chloroform layer was removed and placed into a new 17 mm x100 mm glass tube. Chloroform in the new tubes was evaporated under a stream of nitrogen to dried residue and set aside for silica solid phase extraction (SPE). Silica SPE 100 mg cartridges (Agilent Technologies, Santa Clara, California) were sequentially conditioned with HPLC grade 95% aqueous methanol (1 mL), HPLC grade methanol (1 mL), and HPLC grade chloroform (2 mL). Dried chloroform residue was then reconstituted in HPLC grade chloroform (0.5 mL/g) and washed through an SPE cartridge three times to make sure CTX and CTX-like compounds are trapped. Silica SPE cartridges are well suited for retaining polar molecules, CTX and CTX-like compounds, from nonpolar matrices, such as many of the complex compounds that are found in fish tissue. The SPE cartridge was then washed with HPLC grade 10% methanol in chloroform (1 mL/g) to remove trapped CTX and CTX-like compounds that may be present in fish tissue. 10% methanol and

chloroform solutions for specified fish were dried under a stream of nitrogen to residue and then stored in a - 20˚C freezer until ready for toxin analysis.

In Vitro Neuroblastoma Cytotoxicity Assay (N2a Bioassay)

N2a bioassays are a functional bioassay that can detect bioactive agents that bind to molecular and cellular components to determine toxin groups by mode of action (Rossini, 2005). Since there is no international standard procedure for detection, this bioassay is a desirable method for initial screening for the presence of a multitude of marine biotoxins, as shown in Table 2 (Dickey, 2008; Nicolas et al., 2014). CTX and CTX-like compound screening was conducted using an ouabain-veratridine dependent *in vitro* neuroblastoma (N2a) bioassay. This bioassay has been used to detect voltage gated sodium-channel (VGSC) active toxins as well as non-selective cation channels (NSCC) (Manger et al., 1995; Dickey, 2008). N2a cells were propagated and cultured in RPMI-1640 media supplemented with antibiotics (50 ug/mL streptomycin, 50 units/mL penicillin), L-glutamine (2 mM), sodium pyruvate (1 mM), and heat-inactivated fetal bovine serum (10% v/v) (Dickey, 2008). Cells were harvested for assay at ~80 - 95% cell coverage on the surface culturing flasks. Cells were seeded into sterile 96 well plates at a cell concentration of 35,000 cells/well. Cell viability after exposure represented the bioassay endpoints, which were measured using the colorimetric indicator, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) in a microplate reader (TECAN Sunrise) at a wavelength of 570 nm (Manger et al., 1993; Dickey, 2008). When VGSC or NSCC toxins were detected, full dose-response curves were conducted by serial dilution (8 dilutions) of extracted samples to determine toxin concentration at

which cell viability can be compared to C-CTX-1 reference material to calculate an ID_{50} (Dickey, 2008).

Stable Isotope Analysis ($\delta^{13}C$ and $\delta^{15}N$), Trophic Level Analysis, and Food Web Magnification Factor

Stable isotope analysis of $\delta^{13}C$ and $\delta^{15}N$ were determined in these samples by a collaborative study and reported by Rumbold et al. (2018). Briefly, they haphazardly took 1 – 2 g subsamples from fillets. Tissue samples were placed into a drying oven at a minimum of 60˚C for 48 hour to obtain a dry weight. Dried out samples were then ground by mortar and pestle into a fine powder that were again dried inside a drying oven at a minimum of 60˚C for 24 hours. Powdered dried tissue samples were then shipped to the University of California Davis Stable Isotope Facility, Davis, California, USA to determine and calculate isotope ratios $^{13}C/^{12}C$ and $^{15}N/^{14}N$ and total carbon and nitrogen. Measurements were taken from a continuous flow isotope ratio mass spectrometer (IRMS).

Trophic level of reef fish were conducted using the equation:

$$Trophic\ Level\ consumer = \left(\frac{(\delta^{15}N_{Consumer} - \delta^{15}N_{reference})}{3.4}\right) + 2$$

where the $\delta^{15}N_{reference}$ represents the $\delta^{15}N_{mean}$ of a primary consumer at Tennessee Reef Lighthouse, a frond oyster *Dendostrea frons* (Rumbold et al., 2018). Isotope $\delta^{15}N$ has an on average "enrichment factor" of 3.4 based on laboratory studies on multiple animal species, which increases from one trophic level to the next, and the 2 is the assumed trophic level of the reference primary consumer (DeNiro and Epstein, 1981; Minagawa and Wada, 1984; Jepsen and Winemiller, 2002; Jardine et al., 2006). The 3.4 "enrichment factor" for $\delta^{15}N$, however, has been under scrutiny as being not a valid value because of

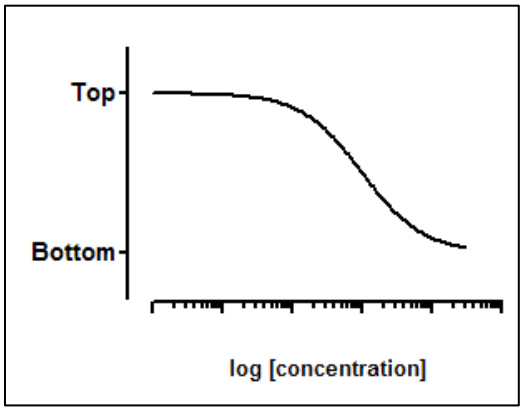
such high variability between dietary preferences and habitat (Vander Zanden & Rasmussen, 2001; Jepsen and Winemiller, 2002; Jardine et al., 2006). Caution, therefore, should be taken when interpreting the calculated trophic level designations. Trophic level calculations were then used to calculate a food web magnification factor (FWMF) for CTX and CTX-like compounds using the equation:

$$FWMF = e^{m}$$

which *m* is the slope of the regression of CTX and trophic level for the species studied. FWMF values for a compound that are greater than 1 is considered to biomagnify in the food web.

C-CTX-1 quantification conducted through GraphPad PRISM 7.04

GraphPad PRISM 7.04 (La Jolla, CA) was used to run a four parameter log(inhibitor) vs response – variable slope model to create a dose dependent curve (Figure 2).



$$Y = Bottom + (Top - Bottom)\frac{(Top - Bottom)}{(1 + 10^{((LogIC50 - X) * Hillslope)})}$$

Figure 2: Log(inhibitor) vs. response – variable slope model. Four variables are taken into account: Top, Bottom, LogIC50, and Hillslope.

This model and equation were used to interpolate C-CTX-1 ng Tissue Equivalency (TE)/g fish tissue for specimens that did not have complete curves due to lack of viable material to be used in the N2a bioassay. Interpolation takes into account all four variables, bottom, top, logIC50, and hillslope, to calculate the best possible "fit" for each curve. C-CTX-1 ng TE/g fish reference material was extracted and quantified at Dauphin Island Sea Lab by Dr. Alison Roberston to be used for quantification of sample fish tissue from the Caribbean region. Fish samples that were used for interpolation were required to have points within the slope of the curve, between 25 – 75% cell viability to ensure acccurate quantification. For fish samples that did have full dose-dependent curves, the IC_{50} was calculated from the model that was then compared to the known concentration of the C-CTX-1 ng TE/g reference material.

Results

Twenty five of the 66 (38%) fish tested positive for sodium channel activity indicative of CTX and CTX-like compounds using N2a methods (Table 3). Twenty three of these fish expressed neuro-toxicity in both sensitized (+O/V) and non-sensitized (-O/V) cell treatments, indicating that a suite of marine biotoxins may be present in fish from Tennessee Reef Lighthouse in the Florida Keys. Monospecific sodium channel toxicity was expressed in only 2 fish represented by one species, *S. barracuda*.

As shown in Table 2, neurotoxicity was detected in multiple reef fish species indicating that CTX and CTX-like compounds can be assimilated regardless of dietary tendencies. Dose dependent sigmodal curves were constructed to quantify C-CTX-1 ng tissue equivalency (TE)/g of fish tissue for the eighteen specimens that had enough

material for this procedure (Table 4). The dose-dependent curves were categorized into one of four curves that were used to tentatively differentiate marine biotoxins dependent upon mode of action (Figure 3). These curves were useful in detecting VGSC activity toxins (CTX and CTX-like compounds) along with non-selective cation channels (NSCC) activity toxins as seen in Figure 3B. The separation between the +O/V and -O/V in this panel indicates a difference in potency, as shown in differences of IC_{50} (17.18 and 92.5 log[extract mg TE], respectively).

Table 2: Fish with common name and scientific name that tested positive presence of CTX and CTX-like compounds.

Common Name	**Scientific Name**	**Toxin Detected (% toxic)**	**Dietary Tendency**
Blue Tang	*Acanthurus coeruleus*	5 (38)	Herbivore
Scrawled Filefish	*Aluterus scriptus*	1 (50)	Omnivore
Porkfish	*Anisotremus virginicus*	4 (44)	Carnivore
Spanish Hogfish	*Bodianus rufus*	1 (100)	Omnivore
Blue Runner Jack	*Caranx crysos*	2 (100)	Carnivore
Rock Beauty Angelfish	*Holacanthus tricolor*	1 (100)	Omnivore
Bermuda Chub	*Kyphosus sectatrix*	2 (67)	Herbivore
Hogfish	*Lachnolaimus maximus*	3 (43)	Carnivore
Black Grouper	*Mycteroperca bonaci*	1 (33)	Carnivore
Gray Angelfish	*Pomacanthus arcuatus*	1 (33)	Omnivore
French Angelfish	*Pomacanthus paru*	1 (50)	Omnivore
Great Barracuda	*Sphyraena barracuda*	2 (18)	Carnivore
Bicolor Damselfish	*Stegastes partitus*	1 (100)	Omnivore

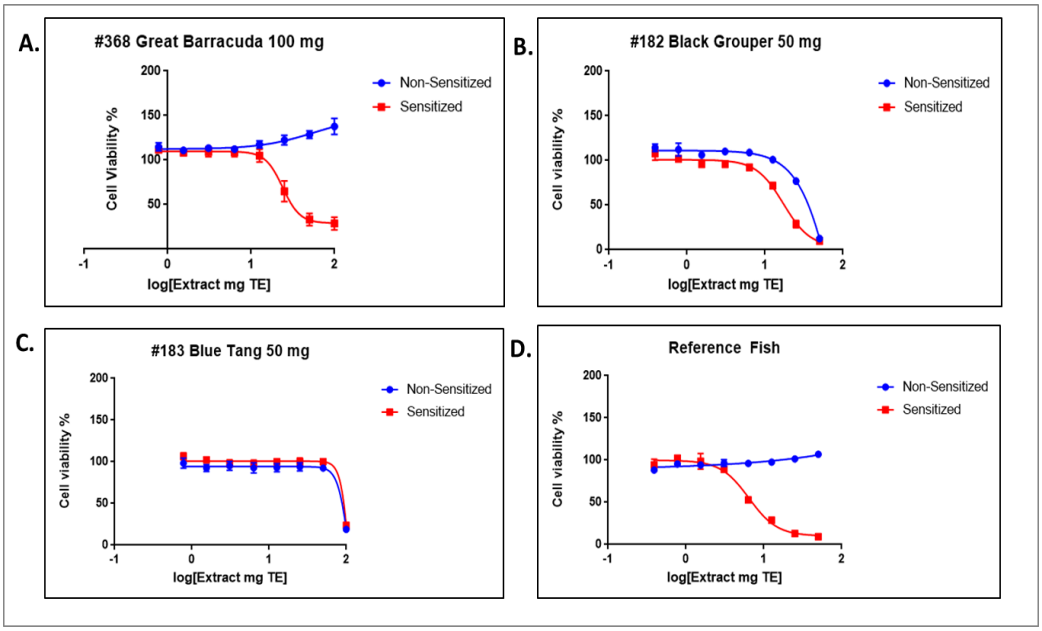


Figure 3: Four responses that were produced from the N2a bioassay; (A) voltage gated sodium channel activity only, (B) voltage gated sodium channel and non-specific cation channel activity together, (C) Non-toxic, and (D) reference fish used as standard.

Average concentrations of C-CTX-1 ng TE/g fish (ppb) could be calculated for two species, *Acanthurus coeruleus* and *Sphyraena barracuda*, and were found to be 0.0878 ± 4.839 and 0.2692 ± 0.8076 C-CTX-1 ng TE/g fish, respectively. Although some species did not have multiple samples tested in this data set, there were some individual samples that were above or near the FDA guidance level of 0.1 C-CTX-1 ng TE/g of fish, such as *S. barracuda Mycteroperca bonaci, Kyphosus sectatrix, Caranx crysos, A. coeruleus, Bodianus rufus, Pomacanthus paru* (Table 3). An interesting finding for *M. bonaci* and *Lachnolaimus maximus*, is that both individual fish were under the legal recreational harvesting size limit for the Florida Keys (406.4 and 609.6 mm, respectively). These individual fish had quantifiable concentrations of CTX, which indicates smaller, younger, and juvenile fish can assimilate and store these neurotoxins to potent concentrations.

Table 3: Fish that were assayed and quantified using individual dose-dependent curves compared to dose-dependent curves of C-CTX-1 reference material (NQ = not quantifiable).

Fish #	Common Name	Scientific Name	C-CTX-1 ng TE/g fish (ppb)	HillSlope Standard Error	Total Length (mm)	Fish Weight (g)
169	Blue Tang	*Acanthurus coeruleus*	NQ	NQ	155	65
183	Blue Tang	*Acanthurus coeruleus*	NQ	NQ	81	12.47
434	Blue Tang	*Acanthurus coeruleus*	0.0789	3.893	148	72.08
435	Blue Tang	*Acanthurus coeruleus*	0.0938	8.556	163	91.64
482	Blue Tang	*Acanthurus coeruleus*	NQ	NQ	235	224.52
483	Blue Tang	*Acanthurus coeruleus*	0.0907	2.069	146	61.96
484	Blue Tang	*Acanthurus coeruleus*	NQ	NQ	186	115.52
188	Scrawled Filefish	*Aluterus scriptus*	0.0618	1.759	400	380
385	Spanish Hogfish	*Bodianus rufus*	0.0856	0.7952	258	271.52
257	Blue Runner Jack	*Caranx crysos*	0.2331	0.2778	311	370
186	Rock Beauty Angelfish	*Holacanthus tricolor*	NQ	NQ	170	150
269	Bermuda Chub	*Kyphosus sectatrix*	0.2755	0.3039	381	935
251	Hogfish	*Lachnolaimus maximus*	0.0441	0.8986	280	320
182	Black Grouper	*Mycteroperca bonaci*	0.2838	0.3347	465	1250
280	Gray Angelfish	*Pomacanthus arcuatus*	NQ	NQ	272	800
259	French Angelfish	*Pomacanthus paru*	0.0808	0.5538	269	715
360	Great Barracuda	*Sphyraena barracuda*	0.3497	0.5442	1016	5100
368	Great Barracuda	*Sphyraena barracuda*	0.1887	1.071	1366	14485

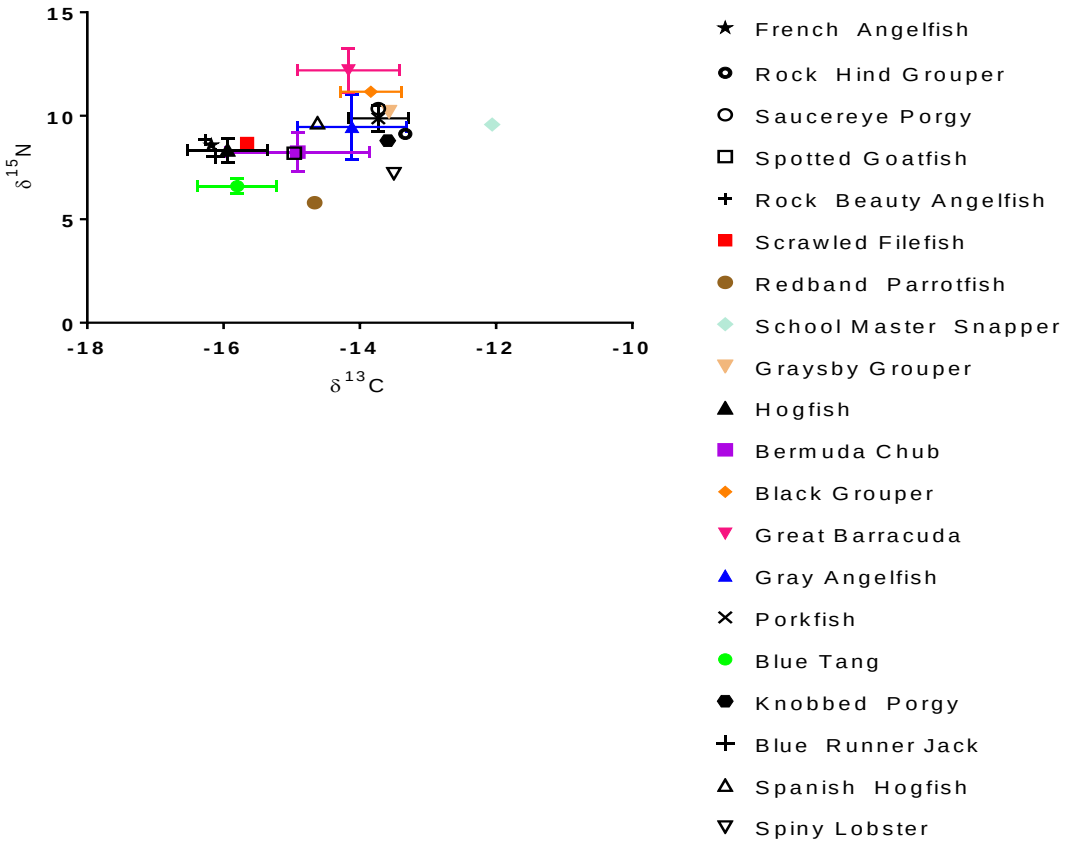


Figure 4: Stable isotope biplot comparing $\delta^{13}C$ and $\delta^{15}N$ for species tested using the N2a bioassay.

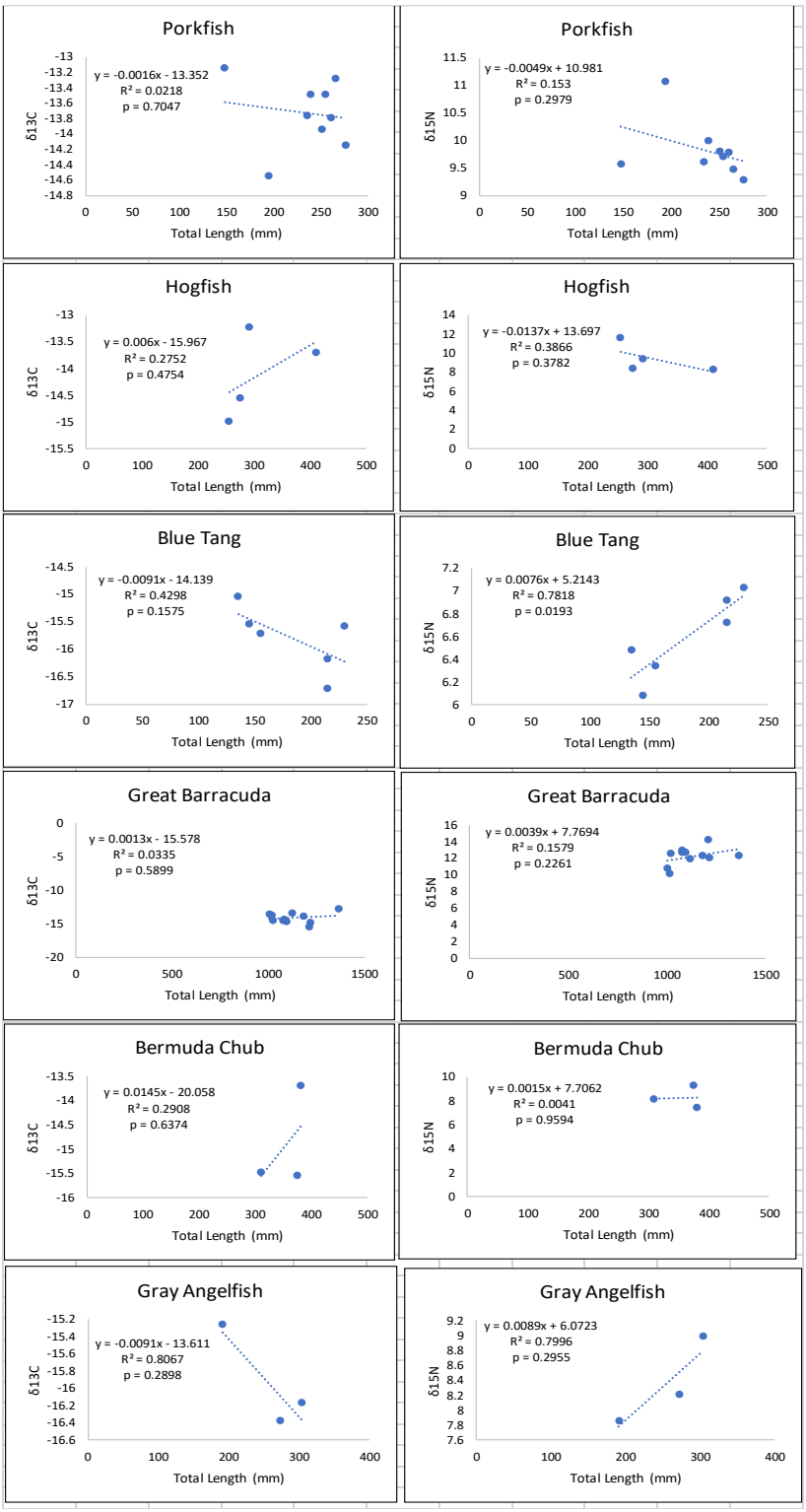


Figure 5: Species specific stable isotope for $\delta^{13}C$ and $\delta^{15}N$ as a function of total length.

As expected, $\delta^{13}C$ and $\delta^{15}N$ increased from herbivorous fish to carnivorous fish and there was fish species variation for both isotopes (Figure 4). Individual species $\delta^{13}C$ and $\delta^{15}N$ were compared to total length to determine ontological shifts in dietary and energy flow in relation to total length as a proxy for age (Figure 5). There were significant relationships between total length and $\delta^{15}N$ for *A. coeruleus* (blue tang) and *M. bonaci* (black grouper) (p-value: 0.019 and 0.025, respectively), indicating that there are possible different nitrogen sources for these fish species over time and area on the reef (Figure 5). Toxin concentrations were also compared to trophic level for quantifiable reef fish species and a significant positive relationship was evident, showing that higher concentrations of C-CTX-1 ng TE/g fish tissue are associated with higher trophic level fish (Figure 6, p = 0.055). FWMF was calculated to be 1.114, indicating that CTX and CTX like compounds at TRL can biomagnify in the food web.

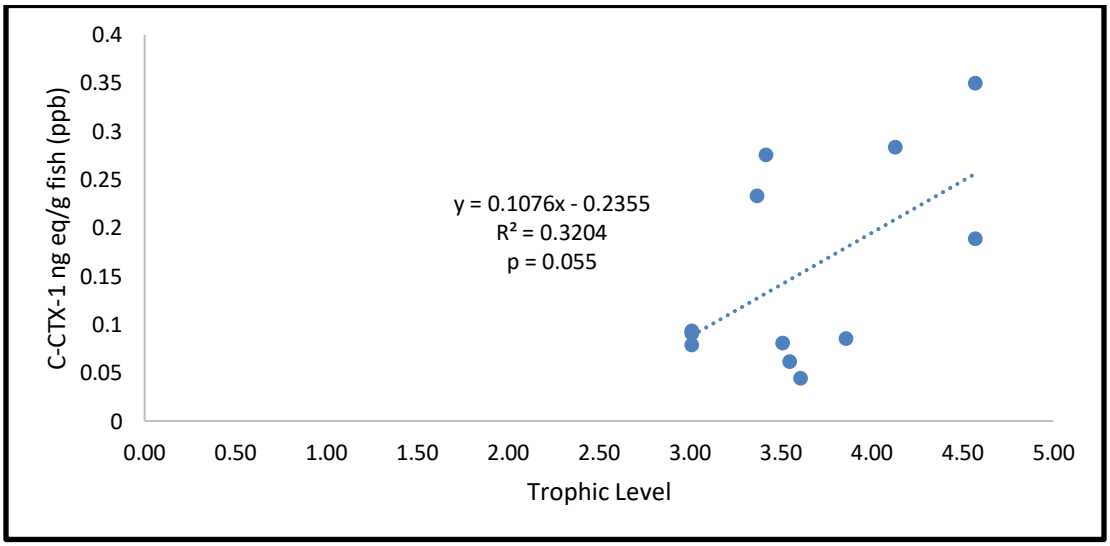


Figure 6: Reef fish trophic level and C-CTX-1 ng TE/g fish regression (p-value = 0.055).

Discussion

Biomagnification of CTXs

This is the first known study examining the presence of CTX-like compounds and other marine biotoxins in multiple fish species from a Caribbean reef (the Florida Keys). The data indicate that these toxins are present throughout the food web starting with herbivorous fishes (i.e. blue tangs, gray angelfish, French angelfish, and damselfish) serving as a major vector to introduce these toxins into the food web. Similar findings were reported in the Pacific region, which indicated that concentrations of CTXs found in herbivores were similar or comparable to many omnivores and carnivores most commonly associated with CFP incidences (Chan et al., 2011; Mak et al., 2013; Gaboriau et al., 2014). These findings were not fully accepted in past literature for the Caribbean region because people would consume herbivorous fish such as parrotfish and no CFP symptoms were reported; however, the data presented herein suggests that Caribbean CTXs follow a similar food web pattern as exhibited by Pacific CTXs (Pottier et al., 2001). The present data also suggest that the concentration of CTXs increases with increasing trophic level with an FWMF > 1 indicating that CTXs and CTX-like compounds do biomagnify (Figure 6, $p = 0.055$). CTX and CTX-like compounds have been found at the highest trophic levels in the marine environment, i.e. in sharks, which have been implicated in severe poisoning events resulting in human comas and deaths according to anecdotal reports (Meyer et al., 2016; Diogène et al., 2017). CTXs seem to follow a similar pattern to other naturally produced, lipid soluble toxins from microalgae, such as okadaic acid, dinophysistoxin-2, yessotoxin, palytoxins, ovatoxins, brevetoxins, microcystins, and β-methylamino-L-alanine (BMAA), which have been found to

biomagnify throughout the food web in marine, aquatic, and terrestrial environments (Cox et al., 2003; Bricelj, et al., 2012; Kozlowsky-Suzuki, et al., 2012; Milandri et al., 2013; Orellana et al., 2017). Along with naturally produced toxins, persistent organic pollutants (POPs), such as polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), polychlorinated nappthalenes (PCNs), polybrominated diphenyl ethers (PBDEs), and methylmercury (MeHg), have also been shown to biomagnify through the marine food web (Nfon et al., 2008; Losada et al., 2009; Mizukawa et al., 2009; Rumbold et al., 2018). The biomagnification efficiencies for naturally produced biotoxins are not well understood and are understudied as compared to POPs, which have utilized stable isotope analysis for quantification.

The use of stable isotope analysis to better understand food web dynamics in a coral reef habitat has been extremely helpful in predicting which species, especially commercially important species, can possibly accumulate CTX-like toxins and other marine biotoxins. Changes in carbon and nitrogen sources can also be a predictor of changing reef fish diets, which vary during fish life history, which seems to be the case especially for herbivorous fish species. The $\delta^{13}C$ and $\delta^{15}N$ values can be quite variable, however, as is demonstrated in Figure 6. $\delta^{13}C$ variations have been documented for herbivores in both marine and aquatic ecosystems, due to plants having low lipid content which affects the synthesis of lipids from dietary carbohydrates resulting in lower $\delta^{13}C$ (Jardine et al., 2006). Additionally, plants can produce nitrogenous defense compounds that are not readily assimilated by herbivores resulting in variable $\delta^{15}N$ (Van Donk, 1997; Vander Zanden and Rasmussen, 2001; Jepsen and Winemiller, 2002; Jardine et al., 2006). Another possible reason for variation in isotope values and CTX concentration for

lower trophic level fish species is because of fish species that are "true omnivores", i.e., those that feed at more than one trophic level, can lead to inconsistencies in the uptake, assimilation, and elimination of nutrients (thereby affecting $\delta^{13}C$ and $\delta^{15}N$ signatures) and contaminants (i.e.CTXs) (Pimm and Lawton, 1978; Tanabe and Namba, 2005; Jardine et al., 2006). A good example of this phenomenon is the Bermuda chub (*Kyphosus sectatrix)*, which was classified as an herbivore feeding on algae, as determined by stomach content analysis (Randall, 1967); however, there are more recent studies indicating that juvenile fish of this species are feeding on zooplankton (Silvano and Güth, 2006). This shift in feeding is interesting to point out because there is some evidence showing that naturally produced toxins (e.g., microcystins, okadaic acid, dinophysistoxin-2, and yessotoxin) have a higher likelihood to biomagnify in zooplankton and zooplanktivorous fish (Ibelings et al., 2005; Kozlowsky-Suzuki et al., 2012; Orellana et al., 2017). Additionally, the toxicokinetics among other naturally produced toxins can be different compared to the properties of CTXs due to differences in chemical structure and the biotransformations that occur in the food web resulting in the need for more research into these compounds.

Using $\delta^{15}N$ and calculated trophic level can be helpful in predicting the presence of CTX-like compounds (e.g., Figure 6). The possible reason that higher trophic level fish may contain higher concentrations of potent CTX-like toxins is the ability of these fish to metabolize and biotransform precursors of these compounds at different (i.e. slower) rates than lower trophic level fish, resulting in an apparent biomagnification up the food web (Vernoux et al., 1985; Lewis & Holmes, 1993; Oshiro et al., 2010; Mak et

al., 2013; Gaboriau et al., 2014; Meyer et al., 2016; Ikehara et al., 2017; Soliño and Costa; 2018).

Trophic level, total length, and weight were originally used to assess ciguatoxicity in reef fish species; however, this approach has been under scrutiny lately as a predictor because such indicators do not seem to be effective in the Pacific region (Caillaud et al., 2010; Gaboriau et al., 2014). Using a FWMF, which uses the calculated trophic level, is an advantageous method in showing the increase of a contaminant (i.e., CTXs) concentration from one trophic level to the next averaged over the entire food web and is comparable to a biomagnification factor (Borgά et al., 2004; Jardine et al., 2006), even though there are some limitations and need for more research as demonstrated above. This method is also helpful for correcting the variations that are observed in $\delta^{15}N$ for organisms throughout all food webs in multiple ecosystems around the world (Fisk et al., 2001; Hobson et al., 2002; Jardine et al., 2006). From the data presented in this study, indicators such as trophic level may be the most useful predictor of CTX biomagnification for the Caribbean region (specifically the Florida Keys); however, caution is warranted due to the small sample size for each species of this study.

N2a bioassay use as a screening tool for marine biotoxins

Rossini (2005) considers a "functional bioassay on the capacity of bioactive agents, including phycotoxins, to bind a molecular component which selectively recognizes the structure of the chemical, and then operationally behaves like its receptor." The N2a bioassay has the capacity to detect suite of bioactive neurotoxins that can be found in a multitude of marine and aquatic environments (Table 4). Currently, there is not a universal and ideal method for the detection of CTXs; however, as shown in this current

study, the N2a bioassay is a useful method for the screening and quantification of CTX and CTX-like compounds (Caillaud et al., 2010). Nevertheless, there are setbacks regarding the N2a bioassay, such as inconsistencies in samples that often contain a mixture of marine biotoxins that can originate from the diverse group of microalgae in marine and aquatic environments. There is a consensus among research groups that a standard detection method needs to be integrated, which likely will include N2a bioassay initial screening followed with high performance liquid chromatography (HPLC) and liquid chromatography dual mass spectrometry (LC/MS/MS) (Dickey, 2008; Caillaud et al., 2010; Nicolas et al., 2014).

Table 4: Marine biotoxins that have been screened and quantification dose-dependent curves were created using N2a cell lines.

Toxin	**Acronym**	**Toxin Source (Nicolas et al., 2014; Visciano et al., 2016)**	**Toxin Mode of Action (EFSA, 2008a; EFSA, 2008b; EFSA, 2009; Nicolas et al., 2014)**	**Reference that have used N2a for Toxin screening**
Okadaic Acid	OA	*Prorocentrum lima* *Dinophysis spp.*	Binds to type 1 and type 2A serine/threonine phosphoprotein phosphatases	Cañete & Diogène, 2008; Ledreux et al., 2012; Sérandour et al., 2012; Bodero et al., 2018
Saxitoxin and its analogs (Neosaxitoxin, Gonyautoxin II, Gonyautoxin II plus III, and Decarbamoylsaxitoxin)	STX, NEO GTX II/III-1 GTX II/III-2 GTX II, dcSTX	*Alexandrium spp.* *Gymnodinium catenatum* *Pyrodinium bahamense*	Binds to Site 1 on VGSC blocking ion conductance preventing depolarization and transmission of the action potential	Manger et al., 1993; Fellett et al., 1995; Manger et al, 1995; Cañete & Diogène, 2008; Melegari et al., 2015; Nicolas et al., 2015
Dinophysistoxin-1 Dinophysistoxin-2	DTX-1 DTX-2	*Dinophysis spp.*	Binds to type 1 and type 2A serine/threonine phosphoprotein phosphatases	Cañete & Diogène, 2008; Bodero et al., 2018
Pectenotoxin-2	PTX-2	*Dinophysis spp.*	Binding site unknown, however, alterations of actin-based cytoskeleton resulting in cell lysis	Cañete & Diogène, 2008; Ledreux et al., 2012; Sérandour et al., 2012; Bodero et al., 2018
Azapiracid-1 Azapiracid-2 Azapiracid-3	AZA-1 AZA-2 AZA-3	*Amphidoma languida* *Azadinium spinosum*	Unknown	Ledreux et al., 2012; Sérandour et al., 2012; Bodero et al., 2018
Yessotoxin 1-Homoyessotoxin	YTX hYTX	*Prorocentrum reticulatum* *Lingulodinium polyedrum* *Gonyaulax spinifera*	Unknown	Bodero et al., 2018
Brevetoxin-1 Brevetoxin-2 Brevetoxin-3	PbTX-1 PbTX-2 PbTX-3	*Karenia brevis*	Binds to site 5 on VGSC causing persistent activation	Manger et al., 1993; Manger et al, 1995; Dechraoui et al., 2005; Cañete & Diogène, 2008;
Palytoxin	PITX/PLTX	*Palythoa spp.* *Ostreposis spp.*	Binds to Na+/K+ -ATPase pump and disrupts ion flow along cell membrane	Cañete & Diogène, 2008; Ledreux et al., 2009; Pawlowiez et al., 2013; Nicolas et al., 2015
Maitotoxin	MTX	*Gambierdiscus spp.*	Binding site unknown, however, increases in Na+ and Ca+ into both excitable and nonexcitable cells	Caillaud et al., 2009; Lewis et al., 2016; Pisapia et al., 2017
Ciguatoxin	CTX P-CTX-1 P-CTX-2 P-CTX-3 C-CTX-1 C-CTX-2 I-CTX-1 I-CTX-2	*Gambierdiscus spp.*	Binds to site 5 on VGSC causing persistent activation	Manger et al., 1993; Manger et al., 1995; Hamilton et al., 2002; Dechraoui et al., 2005; Caillaud et al., 2009; Bottein et al., 2011; Abraham et al., 2012; Ledreux & Ramsdell, 2013; Pawlowiez et al., 2013; Ledreux et al., 2014; Robertson et al., 2014; Lewis et al., 2016; Litaker et al., 2017; Pisapia et al., 2017
Tetrodotoxin	TTX	Pufferfish, and shellfish species	Binds to Site 1 on VGSC blocking ion conductance preventing depolarization and transmission of the action potential	Nicolas et al., 2015; Leão et al., 2018

Ontogenetic Shifts in reef fish life history and its impact on risk of CFP

Other important factors to take into consideration when attempting to predict the likelihood of a particular reef fish species to accumulate CTX-like compounds include fish diet, fish residency time on a reef, and areas of spawning aggregations (Table 5). The base of the trophic transfer of CTXs are the varying toxin loads that are being introduced into the food web by epiphytic *Gambierdiscus* spp., which has been shown to vary by species and strain (Roeder et al., 2010; Lewis et al., 2016; Litaker et al., 2017; Pisapia et al., 2017). Along with toxin loads the variations of the number of *Gambierdiscus* cells over time and among palatable host substrates on reef macroalgae plays an integral role in the introduction of CTXs into the food web (Rains and Parsons, 2015; Parsons et al., 2017). Introductory vector and trophic transfer of CTX-like compounds and other marine biotoxins is through reef fish diets and feeding behavior, which has not been intensely studied in the Greater Caribbean (Randall, 1967). Feeding behavior has been studied for very few species; for example, *Acanthurus coeruleus* (blue tang). Feeding behaviors are at least partially dictated by behavior, such as the aggressive territoriality exhibited by *Stegastes* sp. (damselfish) (Morgan & Kramer, 2005). The life stage and social mode of a blue tang, as well as the reef structure in which these fish reside, also influence their feeding behavior (Morgan & Kramer, 2005). Such behavioral cues could determine if and when blue tangs feed most heavily on reefs where higher densities of toxic epiphytes may be present. As previously mentioned a similar pattern is seen for the reef fish *Kyphosus sectatrix* (Bermuda chub) where juveniles feed more on zooplankton than algae, which indicates shifts in diet when maturing over time (Silvano & Güth, 2006). Changes in fish

feeding preferences with age and life stage are extremely important in understanding the main vectors of CTXs into the food web.

In a related study, the feeding behaviors of herbivorous reef fish including *Acanthurus bahianus* (ocean surgeonfish), *Sparisoma aurofrenatum* (redband parrotfish), and *Scarus taeniopterus* (princess parrotfish) were studied in enclosed versus free-ranging areas, revealing feeding differences that included macroalgae preferences exhibited by these species (Burkepile & Hay, 2008). Along with feeding preference, overall bite rates were observed to be much higher among ocean surgeonfish and princess parrotfish compared to redband parrotfish. These differences, however, did not translate into greater rates of macroalgae consumption by the aforementioned species (Burkepile & Hay, 2008). Additionally, while the ocean surgeonfish and princess parrotfish group may consume the same amount of macroalgae as the redband parrotfish, they may consume higher concentrations and higher potency toxic epiphytic dinoflagellates (i.e. *Gambierdiscus silvae* and *G. excentricus*) due to substrate preferences of such dinoflagellates (Rains and Parsons, 2015).

Reef fish residency is another factor that may influence exposure to CTX-like compounds and other marine biotoxins by fish having either high or low reef residency. The fish *Sphyraena barracuda* (great barracuda), often implicated in CFP cases, has been at the center of a continued debate regarding whether they are predominately a reef or a pelagic fish. Results based on a worldwide examination of mtDNA obtained from barracuda indicates that they are generally a pelagic fish throughout all of the world's oceans (Daly-Engel et al., 2012). On the other hand, there are other studies that have demonstrated great barracuda having high reef residency in specific areas, such as the

Cape Eleuthera, Bahamas, where the median distance traveled by a barracuda ranged from a few hundred meters to over 4 km (O'Toole et al., 2012). Another fish exhibiting a similar behavioral dichotomy is *Caranx crysos* (blue runner jack), which has a 95% daily range of 3082 to 14,333 m^2, indicating that this species can have both high and low reef residency (Brown et al., 2010). Great barracuda and blue runner jack residency times are good examples of the difficulties in predicting the risk of pelagic fish species possibly being chronically exposed to CTXs and CTX-like compounds.

There was a recent study tracking a multitude of fish from a single coral reef location in the U.S. Virgin Islands, including many fish species included in this current study, such as *Calamus calamus* (saucereye porgy), *Acanthurus coeruleus* (blue tang), *Lutjanus apodus* (schoolmaster snapper), *Pseudupeneus maculatus* (spotted goatfish), among others (Pittman et al., 2014). The Pittman et al. (2014) study allowed for quantification of distance covered, or the lack thereof, by these reef fish that could potentially be exposed to toxic epiphytic dinoflagellates. *Balistes vetula* (queen triggerfish), *Lutjanus griseus* (gray snapper), and *Acanthurus chirurgus* (doctorfish) are example species that could possibly be chronically exposed to toxic epiphytic dinoflagellates because they covered less than 4 km during the course of that study (Pittman et al., 2014). A positive and significant correlation was found between total length and distances traveled, in which the largest fish (such as by *Lutjanus analis* (mutton snapper) travelled the farthest (42.2 km; Pittman et al., 2014).

Understanding the movements of economically important fish species, such as *Mycteroperca bonaci* (black grouper), is not only useful for protecting spawning aggregations, but also necessary to know their residency on a reef and potential to have

continual exposures to CTX-like compounds. Lindholm et al. (2005) tracked black grouper in the Florida Keys to determine their site fidelity and found that a majority of these fish were recorded at a single site ranging from 3 to 5 months. Many reef fish base their movements on their spawning events, which is especially important for another economically important species such as *Lachnolaimus maximus* (hogfish). Hogfish in south Florida have very high site fidelity and establish specific spawning territories (areas of hundreds of meters) for mature male hogfish and their harems to patrol and spawn (Lindholm et al., 2006; McBride et al., 2008; Muñoz et al., 2010). Surgeonfish species (*Ctenochaetus striatus*) and grouper species (*Plectropomus leopardus*) have been observed to establish their spawning sites and reef residency on feeding opportunity, which is another possibility to predict their exposure to CTX-like compounds (Zeller, 1998; Claydon et al., 2012). These specific examples show the possible variables and the complexity of the specific life history factors for reef fish species that can lead to the occurrences of CFP cases in tropical and subtropical regions around the world. Fish residency on a specific reef that has high densities of toxic epiphytic dinoflagellates for long periods of time can have a direct effect on which reef fish species could be at high risk of CFP. This exercise also indicates the necessity of further research into the life histories of coral reef fish species to better understand how life history attributes relate to toxin exposure and accumulation.

Table 5: Life history of reef fish that were sampled and tested for CTX-like compounds and other marine biotoxins.

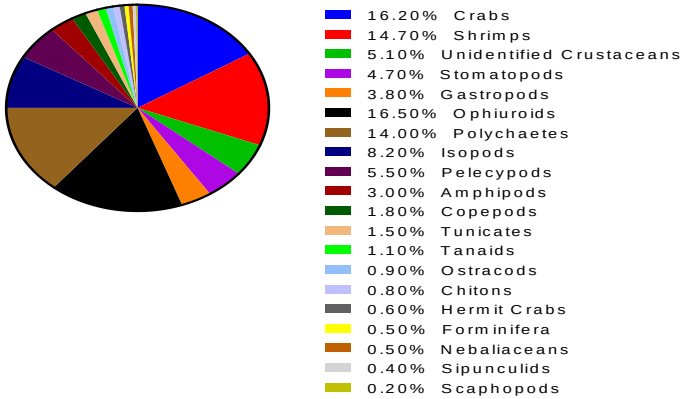
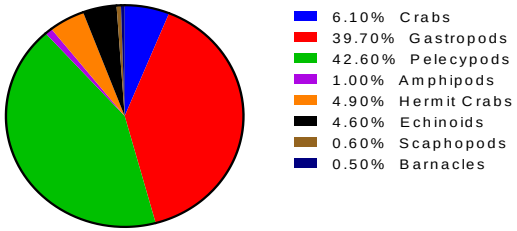
Scientific name	Common Name	Reef Residency	Stomach Content Break Down (Randall, 1967)	Reference
Anisotremus virginicus	Porkfish	N/A		N/A
Lachnolaimus maximus	Hogfish	High Reef Residency		Lindholm et al., 2006; McBride et al., 2008; Muñoz et al., 2010

Table 5: Continued

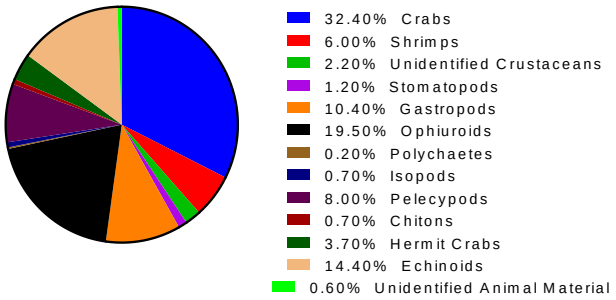
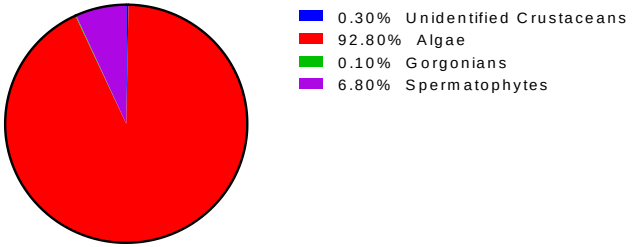
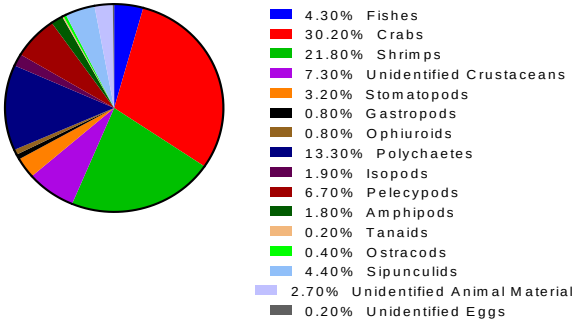
Bodianus rufus	Spanish Hogfish	N/A		N/A
Acanthurus coeruleus	Blue Tang	Medium Reef Residency		Morgan & Kramer, 2005; Claydon et al., 2012; Pittman et al., 2014
Pseudupeneus maculatus	Spotted Goatfish	High Reef Residency		Pittman et al., 2014

Table 5: Continued

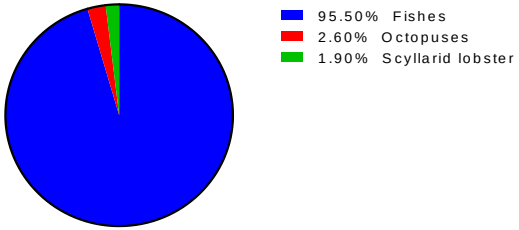
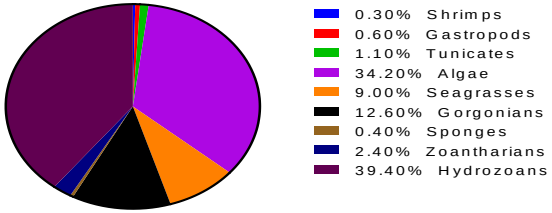
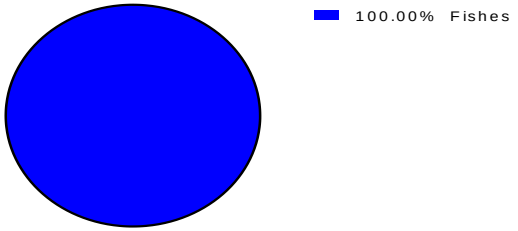
Caranx crysos	Blue Runner Jack	Low and High Reef Residency	N/A	Brown et al., 2010; Dance et al., 2011
Sphyraena barracuda	Great Barracuda	Low and High Reef Residency		Dance et al., 2011; O'Toole et al., 2012; Daly-Engel et al., 2012
Aluterus scriptus	Scrawled Filefish	High Reef Residency		Dance et al., 2011
Mycteroperca bonaci	Black Grouper	Low and High Reef Residency		Jory & Iversen, 1989; Lindholm et al., 2005; Koch, 2011
Stegastes partitus	Bicolor Damselfish	High Reef Residency	N/A	Dance et al., 2011

Table 5: Continued

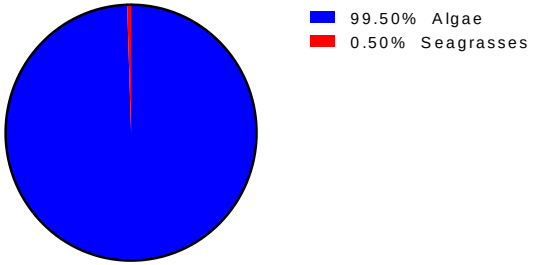
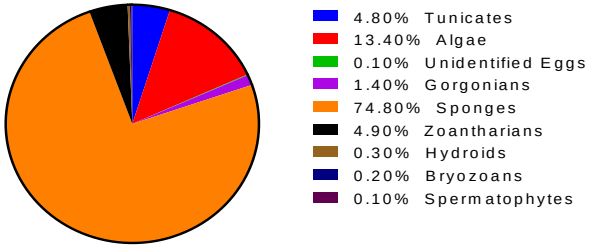
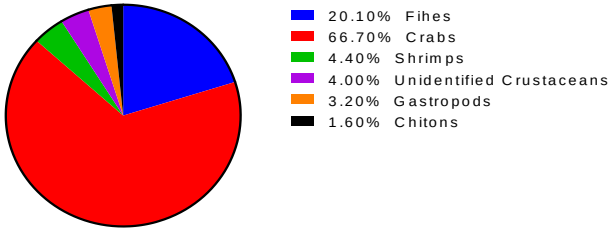
Kyphosus sectatrix	Bermuda Chub	High Reef Residency		Dance et al., 2011
Calamus nodosus	Knobbed Porgy	Medium Reef Residency	N/A	Dance et al., 2011
Pomacanthus paru	French Angelfish	N/A		N/A
Epinephelus adscensionis	Rock Hind Grouper	N/A		N/A

Table 5: Continued

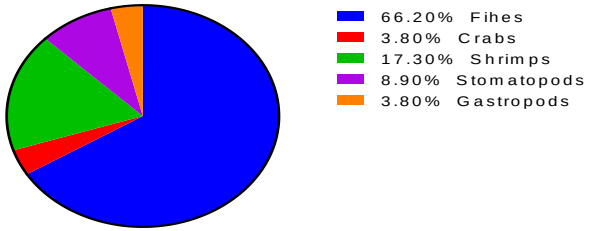
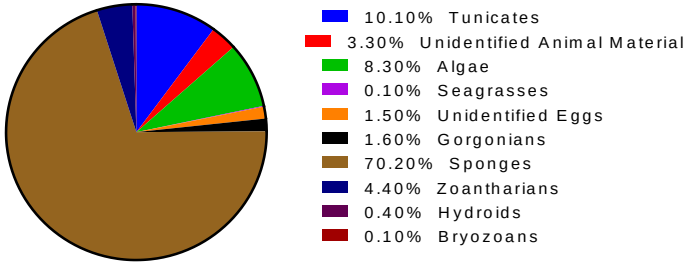
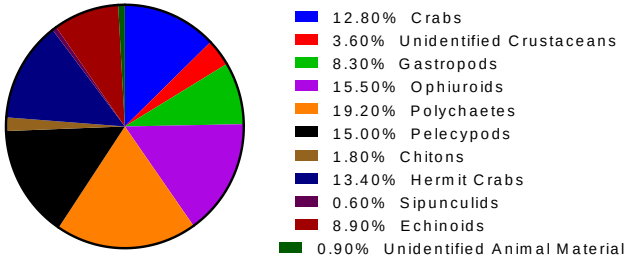
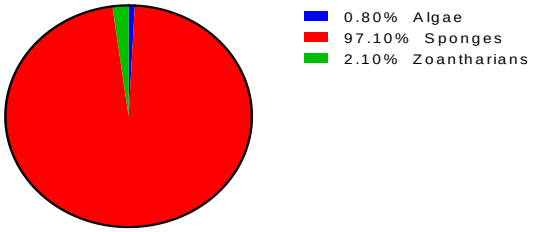
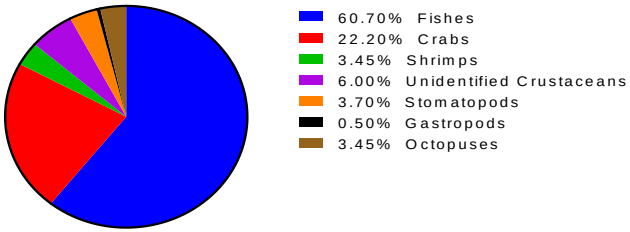
Cephalopholis cruentata	Graysby Grouper	N/A		N/A
Pomacanthus arcuatus	Gray Angelfish	N/A		N/A
Calamus calamus	Saucereye Porgy	Medium Reef Residency		Dance et al., 2011; Pittman et al., 2014

Table 5: Continued

Holacanthus tricolor	Rock Beauty Angelfish	N/A		N/A
Lutjanus apodus	Schoolmaster Snapper	Low Reef Residency		Pittman et al., 2014

South Florida reef fish exposure to CTX and CTX-like compounds

Coral reef fish have been highly exploited, especially in the South Florida and the Florida Keys National Marine Sanctuary (FKNMS) where fishing and tourism have vast economic importance, providing 71,000 jobs and US$6 billion on an annual basis (Johns et al., 2001; Ault et al., 2005). This region is known to have 73 reef fish species along with grunts, jacks, porgies, and hogfish, which are all economically important to recreational and commercial fishing (Ault et al., 2005). The results of the present study document that these local fisheries have a likelihood to assimilate and store CTX and CTX-like compounds in fillet tissue, which in turn can cause CFP illness. Most studies focus on fish as biovectors of these toxins to humans and the resulting human health impacts. Few studies have attempted to establish the negative ichthyotoxic properties of CTXs to coral reef organism health and fitness. Laboratory studies have determined that feeding *Artemia* spp. (brine shrimp) *Gambierdiscus* "*toxicus*" strains (probably a different species based on current knowledge) can cause behavioral abnormalities such as spiral swimming and loss of equilibrium, which would allow these brine shrimp to be easier prey to zooplanktivorous fish (Kelly et al., 1992). Another laboratory study exposed lethal and sublethal doses of purified CTX-1, CTX-2, and PbTx-2 to the fish *Gambusia affinis* (mosquito fish) by adding these toxins to a glass vessel that contained single fish. These exposures showed pronounced opercular movement, inactivity, darkening of the skin, bursts of uncoordinated swimming activity when disturbed, and the loss of righting reflex, which again shows how these toxins can make fish preyed upon easily by higher trophic level fish (Lewis, 1992). On the other hand, there are organisms that have adapted to tolerate pollutants and other naturally occurring toxins, such as

Scomberomorus commersoni (narrow-barred Spanish mackerel) with CTXs, *Salmo gairdneri* (rainbow trout) and *Cyprinus carpio* (common carp) with heavy metals, and *Hemigrapsus oregonensis* (yellow shore crab) and *Hemigrapsus nudus* (purple shore crab) with saxitoxins (Bryan, 1979; Olsson & Hogstrand, 1987; Barber et al., 1988; Hahn et al., 1992). Unfortunately, these adaptations are not present in predators, in this case human populations, which can be exposed to high levels of CTX and CTX-like compound concentrations causing CFP illnesses and symptoms (Bryan, 1979). Individual reef fish that do/may have the adaptation to tolerate CTXs are more likely to survive and persist in coral reef ecosystems, which means that these individuals have an advantage in avoiding predation.

In this study, CTX-like compounds acting on the voltage gated sodium channels (VGSC) were detected in a multitude of different reef fish species in the Florida Keys; however, another class of neurotoxins was also detected using the N2a bioassay (Figure 1). The detection of other neurotoxins leads to the conclusion that there are likely other toxigenic benthic and epiphytic microalgae (i.e. dinoflagellates and cyanobacteria) that are consumed by reef herbivores that are introduced into the food web. Recent studies have indicated that there are CTX-like compounds along with non-selective cation channels (NSCC) toxin compounds present in cyanobacterial mats containing *Trichodesmium* spp., *Oscillatoria* spp., and *Hydrocoleum* spp. (Laurent et al., 2008; Kerbrat et al., 2010; Laurent et al., 2012; Pawlowiez et al., 2013). Another epiphytic dinoflagellate genus, *Ostreopsis*, contains species that produce a potent neurotoxin, a derivative of palytoxin (PLTX), that has been tentatively implicated in CFP cases off Rapa Island of the Australes archipelago, French Polynesia (Pawlowiez et al., 2013). In

addition to these other toxigenic benthic microalgae, the original culprit of CFP cases, *Gambierdiscus* spp., are known to produce a suite of different congeners of ciguatoxins (CTXs) as well as maitotoxins (MTXs). There are many studies that have shown that specific species and strains of *Gambierdiscus* produce a suite of congeners of both of these neurotoxins due to population genetic differences (Roeder et al., 2010; Chan et al., 2011; Kohli et al., 2014; Yogi et al., 2014). As previously mentioned, there are "super-bug" or super producing strains and species of *Gambierdiscus* that reef fish can be exposed to leading to high concentrations of CTXs that may reach levels to cause intoxication in humans over a short period of time (Legrand, 1998; Chinain et al., 2010). For example, the introduction of CTX-like compounds into the reef food web appears to have a ~3 month lag phase between peak *Gambierdiscus* spp. cell densities and CTX-like compounds being detected in reef fish that evidently harm human populations (Chateau-Degat et al., 2005; Clausing et al., 2016a). All of these examples document the need for future studies that can identify all possible benthic microalgae on coral reefs that have been implicated in causing CFP. These current data indicate other lipid soluble neurotoxic compounds can accumulate in fish tissue and be detected using N2a bioassays.

Conclusion

To our knowledge, this is the first study to assess the biomagnification efficiency of CTX and CTX-like compounds across a multitude of reef fish species in the greater Caribbean region. These results suggest that CTX-like compounds are present throughout the food web on a reef in the FKNMS and that other potentially harmful marine biotoxins are also present which merit further risk assessment. This work should

be used as a baseline for future study of CFP in the FKNMS because of its economic importance in South Florida and fisheries based in this region.

Management Issues

The results of this study indicate there is a need for future management plans for specific reef fish species in the Florida Keys due to the presence and biomagnification of CTX-like compounds in local reef fish. CFP cases are predicted to increase around the Caribbean region due to rising sea surface temperatures (SST) with a possible 200 to 400% increase in yearly CFP cases based upon poison center calls during a 10 year study (Llewellyn, 2010; Gingold et al., 2014; Kibler et al., 2017; Walsh et al., 2017). Another important effect of increased SST is the alterations in the metabolism and depuration kinetics in reef fish that are accumulating lipid soluble marine biotoxins, which has been shown to occur for tetrodotoxin and other organic compounds (Hop et al., 2002; Matsumoto et al., 2007; Llewellyn, 2010). Regardless of future trends, CFP can have grave economic consequences on reef fisheries, such as in Tahiti where, in the past, a total of ~3,000 tons of fish per year were not consumed due to the risk of CFP (Bagnis et al., 1993). In the U.S. Caribbean region, there is a concern that an estimated 14% of all reef fish caught in this region should be considered bycatch due to high risk of CFP occurring (Trumble et al., 2006).

Reef fisheries that are targeted for human consumption have high economic value across the globe. With an increase in importation to countries around the world for table fare, as well as for local fish markets found throughout the tropical and subtropical zones, it is evident that there is a high risk of CFP fish being brought to non-endemic regions of the world. Many reef fish are misidentified, or mislabeled, as being a low risk species

(Clua et al., 2011). For the U.S. Caribbean region, i.e., South Florida, Florida Keys, and U.S. Virgin Islands, there is a need for continued monitoring to determine if there are increased incidences of CFP and to see if there is a spread of CFP toxins in the food web to more temperate regions in the Gulf of Mexico and Atlantic Ocean.

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