

Ciguatera: the detection of neurotoxins in carnivorous reef fish from the coast of Cameroon, West Africa

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This work examined 64 large, carnivorous reef fish from the coastal waters of Cameroon for toxicity commonly associated with an incidence of ciguatera fish poisoning. The samples were also subjected to m-DNA analyses to confirm their taxonomic identification. The analyses showed that a subgroup of fish locally referred to as groupers are actually in the snapper family (*Lutjanus* spp.). Extracts from 22 barracuda *Sphyraena barracuda* and 42 snapper *Lutjanus* spp. samples were prepared and examined for the presence of ciguatera-like toxins. Sodium-channel activation was assessed by a sodium-channel-specific bioassay using mouse neuroblastoma (N2a) cells. Extracts were also subjected to chemical analysis via liquid chromatography/mass spectrometry

(LC/MS) to compare the mass of peaks of interest to the molecular weights of fish toxins previously described. Two barracuda and one snapper tested positive for a sodium-channel activator, i.e. presumptive ciguatoxin, in the N2a assay. LC/MS analyses showed that only these three samples contained high-intensity peaks, with masses of 1 222 amu and 1 279 amu. These results represent the first analytical report indicating the presence of sodium-channel-specific neurotoxins in fish from along the coast of West Africa. Given the importance of such marine carnivores to the nutrition and socio-economy of the coastal populace, education and disease management appear to be warranted.

Keywords: Cameroon, ciguatera, *Gambierdiscus*, *Lutjanus* spp., marine toxin, N2a neuroblastoma, neurotoxin, *Sphyraena barracuda*

Introduction

There are ~50 000 reports of ciguatera fish poisoning (CFP) annually, and given the extent of under-reporting and misdiagnosis it is believed that CFP affects at least 500 000 people per year worldwide (Baden *et al.* 1995, Quod and Turquet 1996, Lewis 2001). CFP, manifested by a wide range of clinical symptomology (e.g. gastrointestinal, neurological and cardiovascular disorders), is caused by consuming fish that have accumulated ciguatoxins in their body. Ciguatoxins (CTX) are a class of lipid-soluble polyether neurotoxins that act as a potent activator of sodium ion channels in cellular membranes. It is now generally understood that dinoflagellates of the genus *Gambierdiscus* are the putative producer of gambiertoxins, which are transformed to CTX; these dinoflagellates grow epiphytically on macroalgae in coral reef environments of tropical and subtropical areas. This microbial

biomass is ingested by herbivorous fish, which accumulate the toxin and in turn are preyed upon by carnivorous fish that concentrate, amplify and modify the toxin. Carnivorous reef fish that prey on such herbivores therefore accumulate the toxin, passing it up the food chain and ultimately to humans. It is largely thought that ciguatoxin is oxidised as it is metabolised within the fish, so that the toxin is bioaccumulated and biomodified as it moves up trophic levels. Difficulties in resolving the many implicated unknowns in the CFP process potentially arise from the ephemeral character of CTX production by *Gambierdiscus* spp. Structural analysis requires extremely robust analytical methods owing to the complex molecular structure of CTX and its various congeners, the effective analytical sensitivity required as a consequence of the extreme toxicity of CTX, and its common association with

other phytotoxins that may originate from multiple microalgal species. For such reasons, it is not certain that the clinical symptomology referred to as CFP is caused by a single toxin molecule, i.e. CTX. Despite the potential existence of multiple toxins of varying potency, we here refer to a toxin-eliciting sodium-channel activity as ciguatoxin.

Despite a latitudinal location (4°40' N–2°20' N) characteristic of CFP, scientific studies/analyses of CFP along the tropical coast of Cameroon are non-existent. The closest reported incident of ciguatera in the region was from the Canary Islands (28° N), some 3 000 miles north-west of Cameroon (Perez *et al.* 2005). Because many Cameroon fisheries take large carnivorous reef species that have been implicated in CFP elsewhere, it was considered important to investigate CTX-like toxicity in fish caught there. Interest in studies pertaining to ciguatera have been sparked by coastal surveys showing degradation and coral bleaching, incidents of symptomology commonly associated with CFP after eating reef fish, heavy reliance on carnivorous reef fish by coastal populations, and preference by the local populace for larger carnivorous fish that are generally regarded to have the greatest potential for carrying CFP (Bagnis *et al.* 1982, Gillespie *et al.* 1986, Davin *et al.* 1988, Frenette *et al.* 1988, Bourdeau 1991, Vernoux and Lejeune 1994, Aalbersberg and Sauni 2002). Located in Central West Africa (Figure 1), Cameroon has 360 km of coastline containing a variety of embayments, mangrove forests, coral reefs and sandy beaches. The coast is part of the Guinea Current Large Marine Ecosystem (GCLME). Of the three areas sampled in this work, Limbe and Debunscha are major population centres abutting coral reef coastlines, and Douala possesses both mangrove and sandy beach areas. Coastal fisheries are extremely important to the nutritional and economic health of the local population. Marine fisheries represent the second most important socio-economic activity after agriculture in Cameroon (Enoh *et al.* 1998, Folack *et al.* 1999).

In the work reported here, toxicity was assessed by a sodium-channel-specific N2a bioassay that targets ciguatoxin and polyether-type toxins. In addition to the N2a bioassay, chemical analysis by liquid chromatography/mass spectrometry (LC/MS) was carried out to identify the masses of the targeted compounds for comparison with molecular weights of fish toxins previously described. Given the lack of quantitative analytical information concerning fish from this area, the application of the N2a bioassay in conjunction with LC/MS was intended to determine definitively aberrant sodium-channel activity, i.e. the ciguateric status of these fish, and to evaluate the molecular masses of compounds that caused changes in the sodium-channel activity. Fish extracts were also subjected to the *Artemia* (i.e. *Artemia salina*) bioassay, a non-specific toxicity test. The *Artemia* assay, one of the first non-vertebrate bioassays developed, has been used to assess toxins from plants, fungi and metals, and is considered to be a useful tool for preliminary assessment of toxicity from a wide range of materials.

Material and Methods

Molecular identification of fish

For molecular identification, a small (<0.5 g) piece of muscle tissue was subsampled and stored in 20% dimethyl-

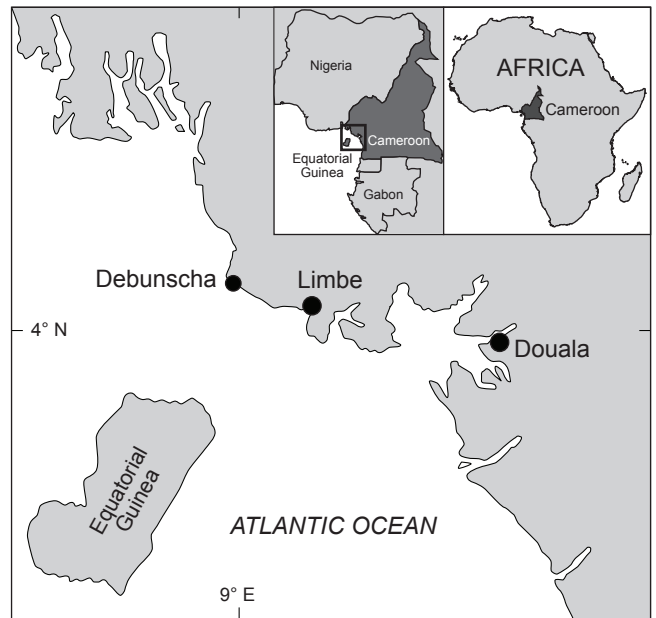


Figure 1: Map showing Cameroon on the central West African coast, extending from 4°40' N to 2°20' N. Fish collections were made at the three areas of Limbe, Debunscha and Douala

sulphoxide-saturated salt buffer (Seutin *et al.* 1991). DNA was extracted using a salting-out protocol adapted from Sunnucks and Hales (1996). Approximately 700 base pairs of mitochondrial cytochrome b were amplified from each sample using the heavy strand primer (5'-GTGACTTGAAAAACCACCGTTG-3') and the light strand primer (5'-AATAGGAAGTATCATTCCGGT TTAGTG-3') designed by Song *et al.* (1998) and Taberlet *et al.* (1992) respectively. An additional 600 bp of the mitochondrial 16S ribosomal RNA gene was amplified in some samples using the primers 16Sar-L (5'-CGCCTGTTTATCAAAAACAT-3') and 16Sbr-H (5'-CCGGTCTGAACTCAGATCACGT-3'), after Palumbi (1996).

The 10 µL PCR reactions consisted of 0.1 U Biolase Taq DNA polymerase (Bioline; Randolph, Massachusetts), 1 × Taq buffer, 0.2 µM of each primer, 200 µM of each dNTP, and 2.0 mM of MgCl₂. PCR amplification on a MyCycler (Bio-Rad; Hercules, California) consisted of initial denaturation at 95 °C for 4 min, followed by 30 cycles of 1 min at 95 °C, 30 s at 55 °C, and 30 s at 72 °C, followed by a final extension at 72 °C for 20 min. Excess oligonucleotide primers were removed with exonuclease I and shrimp alkaline phosphatase (ExoSAP; USB Corp., Cleveland, Ohio) by incubating at 37 °C for 30 min, followed by deactivation at 80 °C for 60 min. PCR products were sequenced on an ABI 3130XL automated DNA sequencer (Applied Biosystems, Foster City, California) at the Hawaii Institute of Marine Biology EPSCoR Sequencing Facility, aligned by eye, and edited using Sequencher v4.6 (Gene Codes Corporation, Ann Arbor, Michigan). BLAST sequence-similarity search (<http://www.ncbi.nlm.nih.gov/BLAST/>) was first conducted to compare the unknown sequences with those in the GenBank (Benson *et al.* 2005) database. Based on those results, a separate phylogenetic tree in PAUP* v4.0b10 (Swofford 2000) was conducted

for each of the genera *Sphyraena* and *Lutjanus* using all available GenBank reference sequences, supplemented with previously identified samples from both genera taken from in-house collections.

Preparation of fish extract

Fish samples for the various toxin analyses were processed as shown in Figure 2. Extracts that caused mortality of neuroblastoma controls without ouabain and veratridine, i.e. evidence of cytotoxicity, cannot be used directly to examine for disruption of the sodium-channel activity that is indicative of ciguatoxins and/or ciguatoxin-like structures. These may be used as a general measure of cytotoxicity. Extracts showing cytotoxicity were further purified and concentrated by repeated processing through the silica columns until cytotoxicity was no longer evident. Once this was achieved, ciguatoxin-like activity was more clearly demonstrated by the cell response caused by the fish extract in the presence of ouabain and veratridine.

To prepare the fish extracts for bioassay testing and LC/MS analyses, ~50 g of muscle from each fish was minced, placed in falcon tubes and lyophilised on a VirTis Freezemobile (VirTis, Gardiner, New York) for 48–72 h. Lyophilised samples were ground into powder, extracted [2:1 v/v of methylene chloride (CH_2Cl_2)] for 5 min under sonification with a solid state ultrasonic FS-9 bath (Fisher, Houston, Texas). The CH_2Cl_2 was then transferred to a glass round-bottom flask. The extraction process was repeated twice with fresh CH_2Cl_2 , and subsequent CH_2Cl_2 volumes were added to the round-bottom flask. Extracts were dried on an R-114 rotary evaporator (Büchi, New Castle, Delaware), and loaded into silica columns (mesh 28–200 μm) to remove oils. Columns were washed with benzene, then eluted with 30% ethyl acetate in benzene. Eluted extracts were re-dried on a rotary evaporator, reconstituted in CH_2Cl_2 , split between two glass vials, and again re-dried. One vial was sent to the Hollings Marine Laboratory (NOS/NOAA, Charleston, South Carolina) for LC/MS analysis, and the other vial retained at the Center for Oceans and Human Health (University of Hawaii, Honolulu, Hawaii) for analysis of sodium-channel activity via the mouse neuroblastoma (N2a) bioassay.

Neuroblastoma bioassay

The N2a bioassay for assessing changes in sodium-channel activity generally follows protocols given by Manger *et al.* (1993, 1995) and Dickey *et al.* (1999).

This bioassay relies on the addition of ouabain and veratridine, which leads to depolarisation of the cellular membranes. This effect is used to elucidate a sodium-channel disruption such as would be caused by the presence of ciguatoxin in fish extracts. All chemicals for the neuroblastoma cell maintenance and bioassay were from Sigma-Aldrich, (St Louis, Missouri), except where specified. A cell suspension of ~200 000 cells ml^{-1} was made using the CCL-131 (ATCC, Manassas, Virginia) in RPMI-1640 cell media, supplemented with 5% fetal bovine serum (Fisher Scientific, Houston, Texas), 2 mM glutamine, 1 mM sodium pyruvate, 50 $\mu\text{g ml}^{-1}$ streptomycin, and 50 units ml^{-1} penicillin. Cells were plated with 100 μl per well at a density of ~20 000 cells

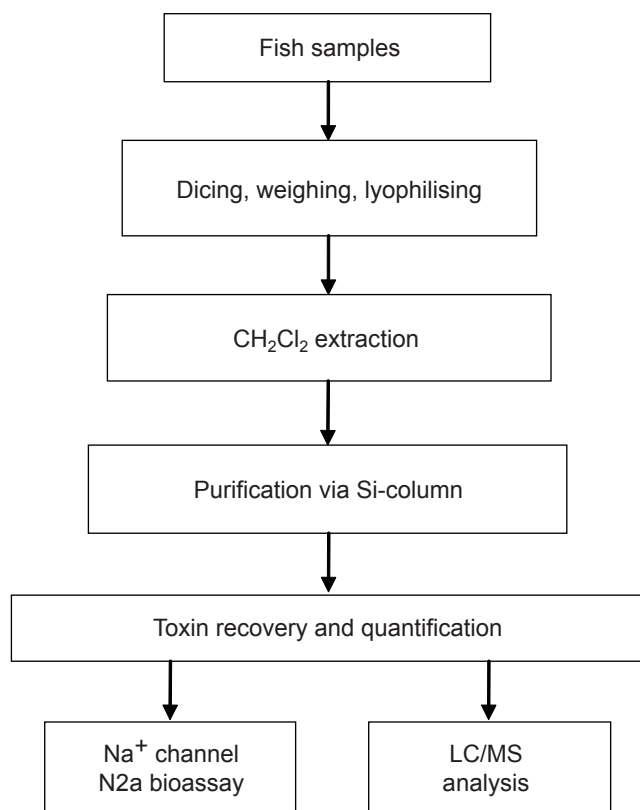


Figure 2: Schematic of fish processing procedures used to produce fish extracts for the various toxicity evaluations performed

in the inner 60 wells of 96-well plates; 100 μl of phosphate-buffered saline (PBS) was added to the 36 perimeter wells. The 96-well plates were then placed in an incubator at 37 °C with 5% CO_2 -enriched and humidified air, and allowed to acclimate overnight. The following day, 500 μl of methanol was added to the vials containing the fish extracts, and the vials were placed in an ultrasonic bath for 3–5 min. Plates were dosed with fish extracts at 1 μl , 2 μl , 3 μl and 4 μl per well, using 10 wells per concentration. Five wells of each concentration also contained 0.3 mM ouabain and 5 μM veratridine, referred to hereafter as O/V. Ten additional wells containing cells-only controls, i.e. no sample and no O/V, were used to represent uninhibited cell growth. Another 10 wells, containing cells plus O/V, were used to represent the baseline decrease in cell viability attributable to the addition of those two chemicals. Additionally, one plate was dosed with 310 pM per well of P-CTX-1 (Lewis, University of Queensland) as a positive control.

Well volumes were brought to 200 μl using the RPMI media, and plates were placed in the incubator overnight. The next day, 20 μl of CellTiter 96 Aqueous One Solution (Promega, Inc., Madison, Wisconsin) were added per well. This solution contains a tetrazolium compound that is bio-reduced by metabolically active cells to produce a colorimetric response. Plates were incubated for 1 h to allow for colour development (used to measure cell viability), then read on a Multiskan MCC/340 Eliza plate reader (Thermo Labsystems, Cincinnati, OH) at 492 nm. Results were

computed and statistically analysed using a Student's *t*-test (Snedecor and Cochran 1980), to identify significant differences between various controls and sample means. Wells containing only cells and fish extracts, i.e. no O/V, were used to first assess the cytotoxicity of the extracts to neuroblastoma cells. Only when significant cytotoxicity was not evident did the interpretive analyses proceed to assessment for aberrant sodium-channel activity. Extracts were deemed to be non-cytotoxic when the means between wells with sample extracts and the control wells were not different ($p > 0.05$). Non-cytotoxic samples were analysed for sodium-channel disruption by comparing readings from wells containing cells plus extract plus O/V against the control wells plus O/V, to determine whether there were significant decreases in cell metabolic activity. Sodium-channel disruption is demonstrated if the viability of the cells exposed to extract and O/V is significantly reduced from that of the cells exposed to O/V alone. Statistical differences were determined from the differences of means of controls ($n = 10$) and samples with extracts ($n = 5$), for the controls with and without O/V. Additionally, once positive samples were identified, the bioassay was repeated with these samples and 15.7 nM of tetrodotoxin, a sodium-channel inhibitor, was added to the wells along with the fish extract and O/V.

LC/MS analyses

Liquid chromatography (LC) analysis was performed on an Agilent (Santa Clara, California) 1100 liquid chromatography

system equipped with a binary pump, chilled autosampler, column compartment and variable wavelength detector. The Gemini 5 μm C18 50 \times 2.0 mm column (Phenomenex, Torrance, California) was eluted at 40 °C using 0.01% acetic acid in water (solvent A) and 0.01% acetic acid in methanol (solvent B) as the mobile phases. A constant flow rate of 0.3 ml min⁻¹ was used. Sample volumes of 5 μl were injected on the column equilibrated in 5% solvent B and 95% solvent A, then held for 10 min under these conditions. Solvent B concentration was then ramped to 100% for another 10 min, after which the composition was immediately returned to the initial condition of 5% solvent B for the remaining 5 min. The elution was monitored by absorbance readings at 254 nm every 1 s.

Mass spectrometry (MS) analysis was accomplished in the positive mode on a Thermo Finnegan (San Jose, California) quantum triple quadrupole mass spectrometer fitted with an electrospray ionisation source (ESI). Spectra were obtained over a mass range from m/z 400 to m/z 1 500.

Artemia bioassay

The *Artemia* bioassay generally followed protocols described by Michael *et al.* (1956), Vanhaecke *et al.* (1981) and Carballo *et al.* (2002). The bioassay was run using the extracted material at a variety of loading levels, e.g. 25 μl , 10 μl , 5 μl and 2 μl extract per 250 μl of seawater. The equivalent wet weight of fish tissue represented by the 25 μl loading is given in Table 1. Fish extracts were dissolved in

Table 1: Summary of fish toxicity results from the N2a cell bioassay. For the positive samples (SN1, C7, C14), the numerical values give the equivalent wet weight of fish tissue that produced that result for the N2a bioassay. Numerical values for all other, i.e. negative, samples give the maximum equivalent wet weight of fish tissue that failed to give a positive reading in the N2a bioassay

Fish ID	Result	Wet weight equivalent (mg)	Fish ID	Result	Wet weight equivalent (mg)	Fish ID	Result	Wet weight equivalent (mg)
<i>Barracuda</i>			<i>Snapper species 1</i>			<i>Snapper species 2</i>		
C1	-	194.5	SN1	+	143.2	SN6	-	57.9
C2	-	209.0	SN2	-	197.9	G2	-	238.3
C3	-	89.1	SN3	-	76.2	G3	-	158.1
C4	-	116.3	SN4	-	123.1	G4	-	61.0
C5	-	181.4	SN5	-	244.9	G5	-	185.5
C6	-	213.3	SN7	-	202.8	G6	-	194.6
C7	+	116.8	SN8	-	108.3	G7	-	149.9
C8	-	265.4	SN9	-	186.7	G8	-	206.5
C9	-	204.3	SN10	-	185.8	G9	-	163.5
C10	-	181.3	SN11	-	70.3	G10	-	245.0
C11	-	18.8	SN12	-	236.9	G11	-	139.3
C12	-	140.5	SN13	-	206.9	G12	-	278.9
C13	-	220.6	SN14	-	188.2	G13	-	112.8
C14	+	18.1	SN15	-	155.3	G18	-	198.3
C15	-	328.7	SN16	-	225.7	G19	-	150.3
C16	-	251.0	SN17	-	88.5	G20	-	250.3
C17	-	187.8	SN18	-	207.5	G21	-	194.5
C18	-	236.8	SN19	-	198.2	G22	-	139.0
C19	-	199.0	SN20	-	191.4	<i>Snapper species 3</i>		
C20	-	197.9				G1	-	212.0
C21	-	125.6				G14	-	242.4
C22	-	179.7				G15	-	174.8
						G16	-	179.9
						G17	-	202.2

methanol. For all samples and loading levels, 10 *Artemia* nauplii were added to each of triplicate glass culture tubes, mixed with extract volumes and allowed to stand for 24 h at 23 °C, after which the tubes were examined for mortalities. Addition of just 25 µl of methanol served as triplicate controls and caused no mortality.

Results

Molecular identification of fish

Table 2 summarises the size data for the four species of large carnivorous fish examined, and shows the areas (Figure 1) where they were collected. 'ID' gives the sample identification numbers used throughout the various processing methods. The 22 barracuda *Sphyræna barracuda* ranged in size from 9 kg to 32 kg, averaged 16 kg, and came mostly from Debunscha. Using the molecular analyses, it was possible to positively assign the 22 barracuda samples to *S. barracuda* (accession no. EU124671) using previously identified samples from Hawaii and comparisons with sequences from nine congeners drawn from GenBank. Collections off Cameroon originally targeted barracudas, snappers and groupers, but m-DNA analyses revealed that some fish identified locally as groupers were in fact two species of *Lutjanus*. Besides the barracuda, the other 42 samples belonged to three closely related genetic groups within the genus *Lutjanus*. In all, sequences were available from just 17 of the ~67 species of *Lutjanus*, and this lack of reference samples

in the GenBank made species-level identification impossible (Meyer and Paulay 2005). However, all 42 samples nested deeply within the genus *Lutjanus* with high support, and were sister to *L. argentimaculatus*, *L. peru* and *L. novemfasciatus* (accession nos DQ444481.1, AY947840 and AY958620 respectively). In the absence of positive reference material, the samples can be grouped into three groups, but can be identified only as *Lutjanus* spp. The average size of *Lutjanus* species 1, 2 and 3 were 8.0 kg, 14.5 kg and 7.2 kg respectively.

Neuroblastoma bioassay

Of the 64 fish examined, two barracudas and one snapper (4.7%) tested positive for ciguatoxins by the N2a sodium-channel-specific bioassay. All three fish (C7, C14 and SN1) were caught off Debunscha. Neither of the barracuda (C7, C14) was as large as the mean size for this group, and some of the negative barracuda were considerably larger. The positive snapper (SN1) was the largest of the *Lutjanus* species 1 group, but was much smaller than fish from the other two *Lutjanus* species groups that were not positive. A positive control of 310 pM of P-CTX-1 demonstrates assay efficacy. Tetrodotoxin utilises an opposite mechanism of action from CTX, by blocking sodium-channel activity in cells. The addition of 15.7 nM of tetrodotoxin in assay caused a reversal of sodium-channel activation, i.e. cell viability increased, so demonstrating the presence of a compound that is sodium-channel-specific for C7, C14 and SN1 fish extracts.

Table 2: Summary of fish data. Fish were collected in January and February 2007, and included barracuda *Sphyræna barracuda*, ID C1–22, and three species of snappers (*Lutjanus* spp.), ID SN1–20 and G1–22, grouped below as snapper species 1, 2 and 3. Asterisks denote the fish that tested positive for ciguatoxin

ID	Location	Fish weight (kg)	Fork length (cm)	ID	Location	Fish weight (kg)	Fork length (cm)	ID	Location	Fish weight (kg)	Fork length (cm)
<i>Barracuda</i>				<i>Snapper species 1</i>				<i>Snapper species 2</i>			
C1	Limbe	11	131.8	SN1*	Dibunscha	10	82	SN6	Dibunscha	8	80
C2	Dibunscha	20	145.3	SN2	Dibunscha	9	82.6	G2	Dibunscha	9	85
C3	Dibunscha	15	136.1	SN3	Dibunscha	5	68.5	G3	Dibunscha	12	84.4
C4	Dibunscha	15	136.1	SN4	Dibunscha	4	62.3	G4	Limbe	17	115
C5	Dibunscha	13	138.5	SN5	Dibunscha	9	87	G5	Limbe	15	93
C6	Dibunscha	11	131.8	SN7	Dibunscha	10	82	G6	Limbe	13.5	82
C7*	Dibunscha	9	116.9	SN8	Douala	7.5	69	G7	Limbe	12	84.4
C8	Dibunscha	15	136.1	SN9	Douala	8.5	81.7	G8	Limbe	11	76.3
C9	Dibunscha	17	146	SN10	Douala	8	80	G9	Dibunscha	9	85
C10	Dibunscha	13	140.7	SN11	Douala	8.5	68	G10	Dibunscha	9	85
C11	Dibunscha	12	131	SN12	Douala	9	70.7	G11	Dibunscha	9	85
C12	Dibunscha	9	116.9	SN13	Douala	7	74.5	G12	Dibunscha	9	85
C13	Dibunscha	11	131.8	SN14	Douala	6	72.7	G13	Dibunscha	9	85
C14*	Dibunscha	15	135.2	SN15	Douala	7.8	74	G18	Dibunscha	32	119
C15	Dibunscha	17	146	SN16	Douala	9.5	81.5	G19	Dibunscha	36	125.3
C16	Dibunscha	14	139.6	SN17	Douala	9	82.6	G20	Dibunscha	14	91.5
C17	Dibunscha	17	146	SN18	Douala	8.5	79.5	G21	Dibunscha	11	85
C18	Dibunscha	16	137	SN19	Douala	7.6	79	G22	Douala	25.5	114.2
C20	Dibunscha	31	179.8					<i>Snapper species 3</i>			
C21	Douala	32	180					G1	Limbe	8	75
C22	Douala	21	156					G14	Dibunscha	7	70.3
								G15	Dibunscha	9	85
								G16	Dibunscha	6	60
								G17	Dibunscha	6	60

Figure 3 shows the N2a assay results for the three positive samples (C7, C14 and SN1), and the results for the P-CTX-1 positive control. All are assigned to the mean of the absorbance values of control wells with cells, no sample and no O/V, representing cell viability on the 96-well plate, untreated. The graphic display of O/V control represents the mean absorbance value of the control wells with cells and O/V, and shows baseline cell death from the addition of O/V. The O/V control value is displayed as a percentage of the cells-only control (~56%). Sodium-channel disruption is demonstrated if the viability of the cells exposed to extract and O/V is significantly reduced from that of the

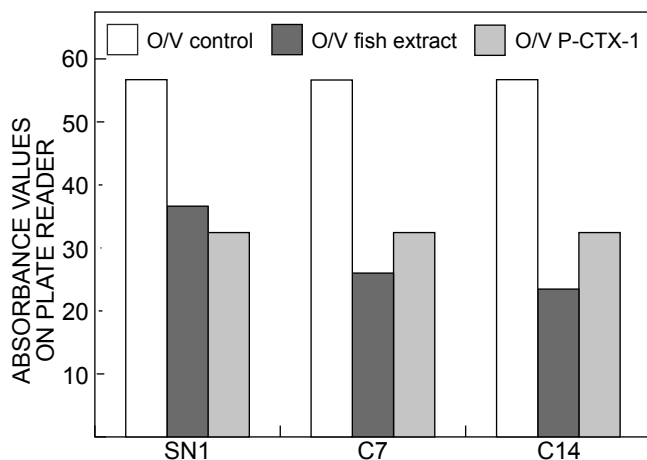


Figure 3: N2a bioassay results for the samples that were positive for sodium-channel disruption along with the results of a P-CTX-1 standard. Baseline cell death caused by the addition of ouabain and veratridine (O/V) is represented at 56%. Sodium-channel disruption is demonstrated if the viability of the cells exposed to extract and O/V is significantly reduced from that of the cells exposed to O/V alone. Cell viability further diminished to 36.6%, 26% and 23.5% with the addition of the three positive samples, SN1, C7 and C14. A 310 pM standard of P-CTX-1 caused cell viability to decrease to 32.4%

cells exposed to O/V alone. Cell viability further diminished to 36.6%, 26% and 23.5% with the addition of the three positive samples, SN1, C7 and C14. A 310 pM standard of P-CTX-1 caused cell viability to decrease to 32.4%. Activity reversal was demonstrated when the absorbance values of the positive fish samples were returned to 76.3%, 84.5% and 82% for SN1, C7 and C14, with the addition of 15.7 nM of tetrodotoxin (data not shown).

LC/MS analyses

The resulting chromatogram for sample C7 is shown in Figure 4; note the distinctive peaks at 1 222 amu and 1 279 amu. Only samples C7, C14 and SN1 showed these molecular fingerprint peaks. Total ion chromatograms of the 1 222 amu and 1 279 amu peaks are shown in Figure 5. Data are not shown for samples C14 and SN1.

Artemia bioassay

Results (data not shown) demonstrated toxicity for 22 fish samples when 25 μ l of fish extract was added to 250 μ l of seawater containing 10 *Artemia salina*. The positive samples consisted of seven samples each of barracuda and snapper species 1, and eight samples of snapper species 2. All other samples were negative. All treatments at three lower extract concentrations, i.e. 10 μ l, 5 μ l and 2 μ l extract per 250 μ l of seawater, failed to show toxicity in any sample. This serves effectively to bracket the actual range where the toxicity threshold lay for this assay.

Discussion

This is the first report of ciguatera in fish from the coast of Cameroon, West Africa (2–4° N). Ciguatera fish poisoning has been documented throughout the subtropical Pacific Islands, tropical Indian Ocean and the Caribbean Sea. Although hundreds of species have been associated with ciguatera throughout the world, jacks, groupers, snappers,

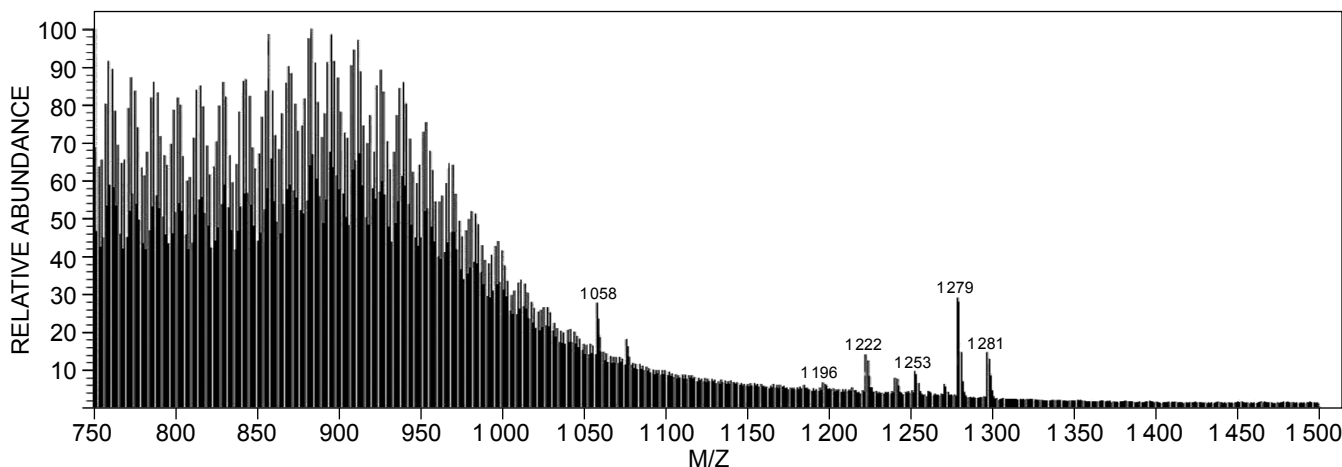


Figure 4: LC/MS spectrum of the bioactive fractions isolated from fish extract C7. Peaks at 1 222 amu and 1 279 amu were isolated in active fractions at every stage of purification for this sample, as well as the other two positive samples C14 and SN1. The isotopic envelope associated with each of the two peaks is also characteristic of the polycyclic polyether toxins

barracuda and mackerels have been most commonly implicated. The sampling for this study targeted these carnivorous species because of their frequent implication with ciguatera fish poisoning elsewhere, and because of their great popularity with the local population. Despite all three fish that tested positive for ciguatera by the N2a assay coming from the Debunsha area, they were not among the largest fish evaluated from this site, which attained weights of up to 36 kg. The barracuda sample C14 (15 kg) was just under the average weight for the group which ranged to 32 kg, and the equivalent wet weight of sample C14 that was evaluated in the N2a assay was among the lowest of any of the samples tested. The snapper sample SN1 was the largest fish of the group at 10 kg, but the equivalent wet weight of sample used in the evaluation was among the lowest of any of the samples tested. That relatively low levels of equivalent wet weight of fish tissue were required to elicit a clearly toxic response (Figure 3) indicates that these fish were especially toxic. Collectively, this indicates that nutritional and foraging aspects of these fish, rather than size alone, are contributing to the accumulation of ciguatoxin.

MS analysis of CTX active fractions demonstrated peaks at 1 222 amu and 1 279 amu (Figure 4). These peaks were found only in the three samples that tested positive by the sodium-channel-specific N2a assay (C7, C14 and SN1). None of the other fish extracts tested demonstrated these peaks. Even after purification, the peaks at 1 222 amu and

1 279 amu remained in the three positive fish extracts, so providing evidence that they were not artifacts. The resonances in the 3–5 μm range were indicative of protons attached to oxygenated carbon, i.e. polycyclic ethers. The two masses exhibited isotopic abundance patterns characteristic of typical polycyclic polyether brevetoxin and ciguatoxin compounds already known (Vernoux and Louis 1997, Lehane and Lewis 2000, Hamilton *et al.* 2002). This information, along with the cytotoxicity data found in the N2a cell lines, were clear evidence that these compounds polycyclic ethers similar to other toxins. In addition, the N2a bioassay, currently with a sensitivity of ~ 6.5 pM, actually paralleled the sensitivity limit for subsequent LC/MS analyses. This is also characteristic of many of the polycyclic ether toxins. Improvements in statistical verification in this method heighten confidence in findings from this bioassay. That these peaks were not evident in any of the other samples, but were the major peaks in the active samples, provides strong circumstantial evidence that the toxin(s) involved is a ciguatoxin-type neurotoxin. It is interesting, though, that the 1 222 amu and 1 279 amu molecular masses are considerably different from the 1 112 amu associated with P-CTX-1 ciguatoxin (Yasumoto and Murata 1990). It cannot be known whether this difference represents a distinct toxin, an African CTX congener, or a structural modification of termini of a (Caribbean) ciguatoxin molecule that might be caused by oxidation (Yasumoto *et al.* 2000, Naoki *et al.* 2001).

Although the *Artemia* bioassay offers non-specific assessment of toxicity, it was included to evaluate its findings in the context of other more robust analyses more specifically targeting ciguatoxin. For the samples that were positive in the *Artemia* bioassay, an average of 81% more equivalent wet weight of fish tissue was evaluated than in the respective samples evaluated in the neuroblastoma bioassay. Because the equivalent wet weights of fish tissue applied in these bioassays were considerably different, the findings are not directly comparable.

This clear finding of ciguatoxin in popular carnivorous fish indicates that there is a health hazard in the area. As is typical for ciguatera studies, the data offer insights to the problem, but do not provide unequivocal directives for preventive management, e.g. 'hot' fishing areas, fish species or fish size. The findings represent merely a basis for educational measures to increase awareness in local communities of the potential hazards of consuming these large carnivorous fish. Similarly, educational efforts within the medical sector that services these communities can further the accurate diagnosis and treatment of the complex symptomatology associated with cases of ciguatera fish poisoning.

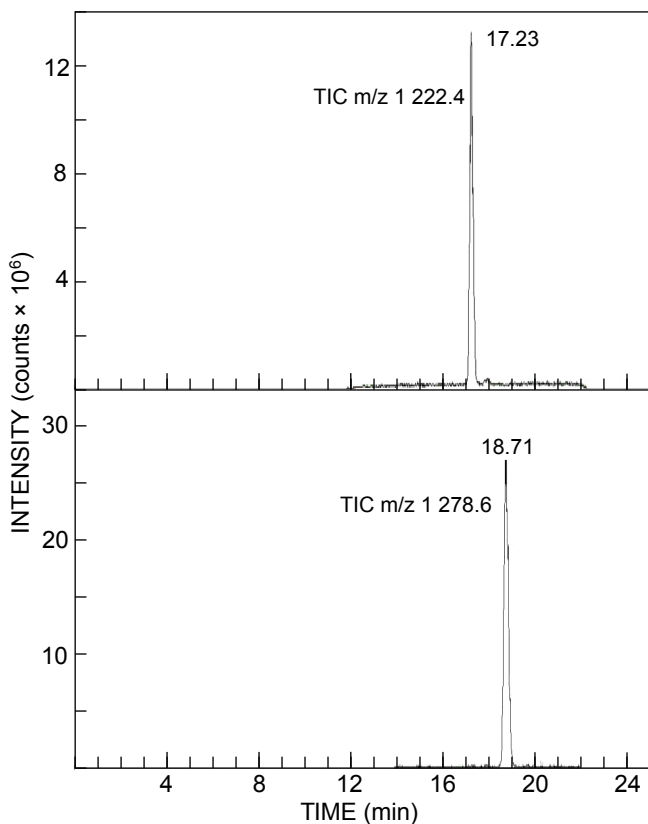


Figure 5: Total ion chromatograms of the peaks isolated from purified bioactive fish flesh extracts. The two masses (1 222 amu and 1 279 amu) do not correlate with any known CTX congeners

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References

- Aalbersberg W, Sauni LF (2002) Marine toxins in the Island Pacific. *University of the South Pacific Institute of Applied Sciences Technical Report 2002/09*: 1–10
- Baden D, Fleming LE, Bean J (1995) Marine toxins. In: deWolff FA (ed) *Handbook of Clinical Neurology: Intoxications of the Nervous System. 2. Natural Toxins and Drugs*. Elsevier, Amsterdam, pp 141–175
- Bagnis RA, Kuberski T, Langier RS (1982) Clinical observations on 3 cases of ciguatera (fish poisoning) in the South Pacific. *American Journal of Tropical Hygiene* **31**: 574–578
- Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Wheeler DL (2005) GenBank. *Nucleic Acids Research* **33**: D34–D38
- Bourdeau P (1991) Ichthyosarcotoxism as ciguatera: a study of ciguatoxic fishes around Saint-Barthelemy and Saint Marteen (French West Indies). In: Fremy JM (ed) *Proceedings of the Symposium on Marine Biotoxins, Paris, France*. CNEVA Publication Series, Maisons-Alfort, Paris, pp 69–74
- Carballo JL, Hernandez-Inda ZL, Perez P, Garcia-Gravalos MD (2002) A comparison between two brine shrimp assays to detect *in vitro* cytotoxicity in marine natural products. *BMC Biotechnology* **2P**: 17–22
- Davin WT, Kohler CC, Tindall DR (1988) Ciguatera toxins adversely affect piscivorous fishes. *Transactions of the American Fisheries Society* **117**: 374–384
- Dickey RW, Jester ELE, Granade R, Mowdy D, Moncreiff C, Rebarchik D, Robl M, Musser S, Poli M (1999) Monitoring brevetoxins during a *Gymnodinium breve* red tide: comparison of sodium channel specific cytotoxicity assay and mouse bioassay for determination of neurotoxic shellfish toxins in shellfish extracts. *Nature Toxins* **7**: 157–165
- Etoh EP, Mbenkun TF, Mbome IL, Folack J (1998) The state of Cameroon's coastal and marine environment. In: Ibe AC, Zabi SG (eds) *GEF'S Large Marine Ecosystem Project for the Gulf of Guinea, State of the Coastal and Marine Environment of the Gulf of Guinea*. Center for Environment and Development in Africa (Ceda), Cotonou, Benin, pp 35–41
- Folack J, Mbome IL, Bokwe A, Tagang A (1999) *Cameroon Coastal Profile*. MINEF/UNIDO/UNDP-GEF, ADE Graphics Industries, Yaounde, Cameroon, pp 1–102
- Frenette C, MacLean JD, Gyorkos TW (1988) A large common-source outbreak of ciguatera fish poisoning. *Journal of Infectious Disease* **158**: 1128–1131
- Gillespie NC, Lewis RJ, Pearn JH, Bourke ATC, Holmes MJ, Bourke JB, Shields WJ (1986) Ciguatera in Australia: occurrence, clinical feature, pathophysiology, and management. *Medical Journal of Australia* **145**: 584–590
- Hamilton B, Hurbungs M, Vernoux JP, Jones A, Lewis RJ (2002) Isolation and characterization of Indian Ocean ciguatoxin. *Toxicon* **40**: 685–693
- Lehane L, Lewis RJ (2000) Ciguatera: recent advances but the risk remains. *International Journal of Food Microbiology* **61**: 91–125
- Lewis RJ (2001) The changing face of ciguatera. *Toxicon* **39**: 97–106
- Manger RL, Leja LS, Lee SY, Hungerford JM, Hokama Y, Dickey RW, Granade HR, Lewis R, Yasumoto T, Wekell MM (1995) Detection of sodium channel toxins: directed cytotoxicity assays of purified ciguatoxins, brevetoxins, saxitoxins, and seafood extracts. *Journal of AOAC International* **78**: 521–543
- Manger RL, Leja LS, Lee SY, Hungerford JM, Wekell MM (1993) Tetrazolium-based cell bioassay of neurotoxins active on voltage-sensitive sodium channels: semiautomated assay for saxitoxins, brevetoxins, and ciguatoxins. *Analytical Biochemistry* **214**: 190–194
- Meyer CP, Paulay G (2005) DNA barcoding: error rates based on comprehensive sampling. *Public Library of Science, Biology* **3**: e422
- Michael AS, Thompson CG, Abramovitz M (1956) *Artemia salina* as a test organism for bioassay. *Science* **123**: 464–465
- Naoki H, Fujita T, Cruchet P, Legrand AM, Igarashi T, Yasumoto T (2001) Structural determination of new ciguatoxin congeners by tandem mass spectrometry. In: DeKoe WJ, Samson RA, Van Egmond HP, Gilbert J, Sabino M (eds) *International IUPAC Symposium on Mycotoxins and Phycotoxins*. Ponsen & Looyen, Wageningen, the Netherlands, pp 475–482
- Palumbi S (1996) Nucleic acids II: the polymerase chain reaction. In: Hillis MC, Moritz C, Maple BK (eds) *Molecular Systematics*. Sinauer Associates, Sunderland, pp 205–248
- Perez JL, Luzardo O, Brito A, Cabrera M, Zumbado M, Carranza C, Angel A, Dickey R, Boada L (2005) Ciguatera fish poisoning, Canary Islands. *Emerging Infectious Diseases*. **11**: 1981–1982
- Quod JP, Turquet J (1996) Ciguatera in Reunion Island (SW Indian Ocean): epidemiology and clinical patterns. *Toxicon* **34**: 779–785
- Seutin G, White BN, Boag PT (1991) Preservation of avian blood and tissue samples for DNA analyses. *Canadian Journal of Zoology* **69**: 82–90
- Snedecor GW, Cochran WG (1980) *Statistical Methods* (7th edn). Iowa State University Press, Ames, Iowa
- Song CB, Near TJ, Page, JM (1998) Phylogenetic relations among percid fishes as inferred from mitochondrial cytochrome b DNA sequence data. *Molecular Phylogenetic Evolution* **10**: 343–353
- Sunnucks P, Hales DF (1996) Numerous transposed sequences of mitochondrial cytochrome oxidase I–II in aphids of the genus *Sitobion* (Hemiptera: Aphididae). *Molecular Biological Evolution* **13**: 510–524
- Swofford DL (2000) PAUP*: phylogeny analysis using parsimony (*and other methods). Version 4 Software. Sinauer Associates, Sunderland
- Taberlet P, Meyer A, Bouvet J (1992) Unusually large mitochondrial variation in populations of the blue tit *Parus caeruleus*. *Molecular Ecology* **1**: 27–36
- Vanhaocke P, Persoone G, Claus C, Sorgoloos P (1981) Proposal for a short-term toxicity test with *Artemia* nauplii. *Ecotoxicology Environmental Safety* **5**: 382–387
- Vernoux JP, Lejeune J (1994) Ciguatera in the French West Indies. *Memoirs of the Queensland Museum* **34**: 631–638
- Vernoux JP, Lewis R (1997) Isolation and characterization of Caribbean ciguatoxins from the horse-eye jack (*Caranx latus*). *Toxicon* **35**: 889–900
- Yasumoto T, Igarashi T, Legrand AM, Cruchet P, Chinain M, Fujita T, Naoki H (2000) Structural elucidation of ciguatoxin congeners by fast-atom bombardment tandem mass spectrometry. *Journal of the American Chemistry Society* **122**: 4988–4989
- Yasumoto T, Murata M (1990) Polyether toxins involved in seafood poisoning. In: Hall S, Strichartz G (eds) *Marine Toxins: Origin, Structure and Molecular Pharmacology*. American Chemical Society, pp 120–132