Ciguatera poisoning trace-back in Europe leads to a novel ciguatoxin-3C group characterization from the Indian Ocean

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Article

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Abstract

The consumption of seafood containing marine biotoxins called ciguatoxins (CTXs) can result in ciguatera poisoning (CP), a globally prevalent seafood-born human illness. Since 2015 isolated and mass outbreaks of CP have occurred along the southwestern coast of India, however, no attributable CTXs have yet been identified. Herein, several CTXs are described in an often marketed snapper species (*Lutjanus bohar*) from southwest India. CTX3C-group compounds were identified by LC-MS/MS with a toxicity range of 0.79-5.39 ng CTX3C equivalent (eq.) g^{-1} wet tissue eq., as determined by an *in vitro* cell (Neuro-2a) assay. Samples investigated were part of a 7,000 kg international shipment of frozen snapper product imported into the European Union and subsequently implicated in a 2020 CP outbreak in the Netherlands. The identification of CTX3C-group toxins in fish originating from coastal India suggests a re-evaluation of the current understanding of CTXs associated CP with seafood from the Indian Ocean region.

Introduction

Seafood plays an important role in meeting rising global food requirements and is one of the most frequently traded food commodities worldwide. Ciguatera poisoning (CP) is a serious food-borne illness that follows the consumption of seafood containing ciguatoxins (CTXs). Globally, tens of thousands of people are estimated to suffer CP annually, with symptoms that include, but are not limited to, gastrointestinal, neurological, and cardiovascular symptomology (as reviewed in Friedman, et al. [1]). Under- and de-centralized CP case reporting or undiagnosed cases and difficulties in toxin identification are commonly cited problems restricting the accurate accounting of CP [1–4]. CTXs are potent neurotoxins produced by microalgae in the genera *Gambierdiscus* and *Fukuyoa*. CTXs have been found in marine animals from various food webs and habitats in tropical, subtropical, and (some) temperate zones as reviewed in Chinain, et al. [5,]FAO and WHO [6,]Tester, et al. [7]. Animals acquire CTXs through their diet (i.e., biomagnification), and following their ingestion, the toxins are incorporated throughout the consumer's body. CTXs in seafood products are organoleptically undetectable and resistant to cooking, freezing, or general food preparation techniques [8]. CTX detection at human health-relevant concentrations (e.g., 0.01 µg CTX1B equivalents (eq.) per kilogram of tissue as recognized by the US Food and Drug Administration [8,9]) from complex food or biological matrices (including various animal tissue types) necessitates sensitive laboratory equipment (e.g., liquid chromatography-tandem mass spectrometry (LC-MS/MS)) operated by trained personnel. Despite these recognized detection, prevention, and epidemiolgical complexities, regulations are in place to attempt to safeguard consumers from products containing CTXs. Within the European Union's (EU) jurisdiction, products containing CTXs must not be placed on the market [10–12]. In endemic and non-endemic regions efforts to manage CP are based on the managing authority's historic knowledge or association with CP and generally include harvest restrictions by location, species, sizes (weight or length), or some combination thereof [9,13–17]. According to Article 4 of 2000/104/EC, the label of any fishery products on sale must contain the commercial name of the species, the production method (catch method), and the catch area. This accurate product information helps provide the

consumer with traceability assurances to ensure the quality and safety of food products (i.e., species and regional association with CP). However, CTX contaminated products have unwittingly been distributed globally by the international seafood trade as evidenced by the reoccurrence of imported seafood products resulting in CP outbreaks [18–20].

One challenge to CTX analysis stems from the chemical diversity of CTXs where over 30 congeners have been identified to date [6], whereas only two CTX standard substances are commercially available, CTX1B and CTX3C (both are from the group associated with the Pacific region). To date, four structural groups of CTXs have been described among three Oceanic basins: CTX4A and CTX3C for the Pacific region, C-CTX for the Caribbean, and I-CTX for the Indian Ocean region. Amongst these groups, I-CTXs are the least understood, lacking a known chemical structure, toxicity, or recognized source. A recent review by Habibi, et al. [21] identified some of the data gaps, vectors, and problems facing the Indian Ocean for CP. Fatalities and mass poisonings of over 200 people have been associated with CP in the Indian Ocean basin [22,23], emphasizing that closing the knowledge gaps regarding CTXs originating from the Indian Ocean basin remains a critical issue.

Generally, CTXs are food contaminants without validated detection methods [6,10], most CTXs lack a complete understanding (chemically, biologically, and ecologically), and only two have a guidance limit (i.e., US FDA guidance levels of 0.1 µg C-CTX-1 eq. per kg and 0.01 µg CTX1B eq. per kg). These problems can be further exacerbated when fish are improperly labeled by species or catch regions [20,24]. Without accurate identification and accountablity, fish that may have undergone management scrutiny can bypass the frequently used routine controls (i.e., species or regional restrictions) for the investigation of CTXs with a guidance limit. Investigations employing a targeted focus using *a priori* assumptions based on a purported species or region with regionally associated CTX compound(s) (i.e., C-CTX-1 in the Caribbean or CTX1B in the Pacific), can miss seafood products containing unknown or undetected CTXs (when not used in tandem with a broad compound type untargeted approach e.g., a bioassay), ultimately resulting in a potential outbreak of CP [25].

Only a handful of CP cases have a clinical diagnosis and even less of these have a meal remnant available from which to conduct a toxin contaminant investigation. To fill existing data gaps for CP, a complete account of the events surrounding the CP case is idealy required, including a medical diagnosis, toxicological investigation of the meal remnant for the attributable CTX analogue with an ascribed toxin value, species authentication for the causative organism, and traceback to the harvest location. The correct attribution of the responsible species, the source region, and compound(s) involved in a CP outbreak are important steps for taking effective follow-up actions and conducting research to address human health (e.g., with physicians for consumer exposure), monitoring (e.g., groups for food control, CTX chemical research, and seafood processors), or environmental factors (e.g., food web trophic toxin transfer investigations, environmental contaminants, environmental factors driving toxin production).

Historically, CP symptomology and CTX molecular descriptions were associated with an Ocean basin, as reviewed in Friedman, et al. [1],FAO and WHO [6]. In the Pacific region symptoms are predominatenly

neurological, in the Caribbean Sea gastrointestinal symptoms are more common, and in the Indian Ocean fish have been more frequently contaminated by lethal levels of toxin and have on occasion reported symptoms of hallucinations and mental depression [26–28]. Herein we provide comprehensive details on an internationally traded seafood lot that was responsible for an outbreak of CP in the Netherlands in 2020. CTXs were investigated in *Lutjanus bohar* originating from the Indian Ocean. This description includes an account of the outbreak, trace-back to the harvest region, and CTX analysis by both an *in vitro* bioassay method and LC-MS/MS; based on available portions of the commercial product in question. The Indian Ocean basin currently suffers from a paucity of data regarding CTXs and CP descriptions. The identification of CTXs in a commercial species can fill a critical data gap regarding attributable CTXs affecting the region, which has been the source of ongoing isolated, mass, and international outbreaks of CP.

Methods

Collection of additional material in Germany related to a CP outbreak

According to the Rapid Alert System for Food and Feed (RASFF) notification number 430888, with reference number 2020.2254 [29], a food poisoning alert notification regarding a serious human health risk for ciguatera poisoning was sent on May 29th 2020. This was in connection with fish, and products thereof, under the name 'Darnes de vivaneau – frozen red snapper steaks (*Lutjanus bohar*)' with the associated lot number 629/2017-08.

Resulting from this alert, two sealed consumer packages from the responsible lot number 629/2017-08 were collected on June 2nd 2020 in the German cities of Bonn and 75 km away in Mönchengladbach. The collected product was packaged on May 8th 2017 and was marked with a best-by date of May 7th 2019. The two sealed bags were received frozen and in good condition at the German Federal Institute for Risk Assessment (BfR) in Berlin, Germany for CTX analysis and contained seven portions of fish (4 and 3 pieces per bag, samples 1 to 7).

Analysis of material

Materials and reagents

All cell line work was performed in a Class II microbiological safety cabinet (model Claire® B-3-160, Berner International GmbH, Elmshorn, Germany). Two CB-60 incubators from BINDER GmbH (Tuttlingen, Germany) were used throughout this study; one dedicated for culture maintenance, and one exclusively for assay-related activities, both incubator conditions (37 °C and 5% CO_2 atmosphere) were the same. Consumables including serological pipettes, C-Chip disposable Hemacytometer, filter capped culture flasks, ninety-six-well polystyrene plates (Corning™ 3596), methanol, *n*-hexane, chloroform, and water (HPLC grade) were purchased from Fisher Scientific GmbH (Schwerte, Germany). Bond Elute silica (SI) solid-phase extraction (SPE) cartridges (3 mL, 500 mg) and Chromabond EASY SPE cartridges (3 mL, 200 mg) were obtained from Agilent Technologies (Waldbronn, Germany) and Macherey Nagel (Düren,

Germany), respectively. Mouse (*Mus musculus*) neuroblast type cells, cell line Neuro-2a (ATCC® CCL-131™) were purchased from the American Type Culture Collection (LGC Standards GmbH Wesel, Germany) from the lot numbered 63649750, which was frozen February 24th 2016 at passage number 184 and modified according to Loeffler, et al. [30]. Culture media and supplements (heat-inactivated fetal bovine serum (FBS), Roswell Park Memorial Institute (RPMI-1640), glutamine, sodium pyruvate, penicillin-streptomycin, 10X Trypsin-EDTA) and reagents for N2a-assay (i.e., ouabainoctahydrate, veratridine hydrochloride, phosphate-buffered saline, dimethyl sulfoxide, HPLC grade water, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)) were obtained from Sigma-Aldrich Chemie GmbH (Munich, Germany). Phosphate Buffered Saline (PBS) was prepared fresh as follows: 16 g NaCl, 400 mg KCl, 2.3 g Na_2HPO_4, and 400 mg KH_2PO_4 were dissolved in 2 L of HPLC grade water; the solution was autoclaved and stored at 4°C. Trypsin (2.0 mL in 38 mL PBS) was used to remove the cells from the culture flask (e.g., for passaging or plating). MTT stock solution was prepared by dissolving 500 mg MTT in 100 mL PBS. Methanol standard solutions of CTX1B (4 µg mL^{-1}), 52-*epi*-54-deoxyCTX1B (i.e., P-CTX-2, 1 µg mL^{-1}), and 54-deoxyCTX1B (P-CTX-3, 2 µg mL^{-1}) were purchased from Professor R. J. Lewis (The Queensland University, Australia, prepared November 2005). CTX3C (100 ng, lot APK4222 and TWJ6482) were purchased from FUJIFILM Wako Chemicals Europe GmbH (Neuss, Germany) and reconstituted in 1 mL methanol. Solutions were stored in glass vials at − 20°C.

DNA barcoding

One sample from each bag was selected for species authentication through DNA barcoding. DNA for the species identification was extracted according to the standard CTAB (Cetyltrimethylammonium bromide) protocol DIN EN ISO 21571:2013-08 [31]. DNA barcoding was performed according to DIN CEN/TS 17303:2019. Cytochrom b (Cytb) barcoding region was amplified with primers L14735 (5'-AAAAACCACCGTTGTTATTCAACTA-3') and H15149ad (5'-GCICCTCARAATGAYATTTGTCCTCA-3') in 25 µL reaction tubes in a Mastercycler gradient cycler (Eppendorf, Hamburg, Germany). Amplicons were sequenced by Eurofins Genomics (Ebersberg, Germany). Sequences were blasted against the genetic sequence database GenBank® of the National Center for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST).

Sample extraction and purification

Muscle tissue (5 g) was excised from each sample, without bones or skin, to facilitate the CTX extraction process. The tissue samples were processed for toxin extraction using previously published methods by Dickey [32] for the N2a-MTT assay and Spielmeyer, et al. [25] for LC-MS/MS. Briefly, for the N2a-assay the muscle tissue was homogenized by ultra turrax and extracted twice with 15 and 10 mL acetone, respectively. The extract was dried under a stream of nitrogen at 40°C. The residue was reconstituted in 5 mL of methanol/water (4:1, v/v) and defatted twice with 5 mL *n*-hexane. The *n*-hexane was discarded, and the aqueous methanol was reduced to dryness under a stream of nitrogen at 40°C. The dry residue was reconstituted in 5 mL HPLC-grade water and CTXs were extracted twice with 5 mL chloroform. The organic extracts were combined, dried, reconstituted in 50 µL chloroform, and applied to a pre-conditioned

(methanol/water 95:5 (v/v), methanol, and chloroform) Bond Elute SI cartridge. The glass vessel was rinsed three times with 200 µL chloroform and the rinse solvent was applied to the column. The cartridge was washed with one column volume of chloroform. Elution was carried out with two column volumes of methanol/chloroform 1:9 (v/v). The eluate was dried and reconstituted in 1 mL methanol. Sample extracts were stored in glass vials at -20°C until usage.

For LC-MS/MS analysis, 5 g tissue was enzymatically decomposed by papain. Extraction was performed using acetone, saturated sodium chloride solution, and ethyl acetate. After washing with saturated sodium chloride solution, the raw extract was reduced to dryness and reconstituted in 80% methanol. Defatting was performed in three steps with *n*-hexane, *n*-hexane after the addition of saturated sodium carbonate, and *n*-hexane after the addition of citric acid solution. Clean-up of the defatted sample was conducted by reversed-phase and normal-phase SPE. The two fractions of the normal phase SPE (filtrate and eluate) were reduced to dryness, reconstituted in 500 µL methanol, and transferred into glass vials. Samples were stored at -20°C before analysis.

Toxicity evaluation by *in vitro* Neuro-2a cytotoxicity MTT-assay

A semi-quantitative *in vitro* Neuro-2a cytotoxicity MTT-assay was used to investigate the sample extracts to determine their composite cytotoxicity, based on the methods described in Manger, et al. [33], Dickey [32], and with cell line modifications as described in Loeffler, et al. [30]. Sodium channel-specific toxin activity was measured by mouse (*Mus musculus*) neuroblastoma (N2a) cells and was dependent on the addition of ouabain (O) and veratridine (V) (OV+). These compounds were used to sensitize the cells for the detection of sodium channel-specific effects. Conversely, N2a cell cytotoxicity that results from another mode of action (other than sodium channel activation) was evaluated using non-sensitized cells (OV-). The composite cytotoxicity response to all sodium channel toxins contained in the sample was conducted with a full dose-response curve (8-dilutions) using a sample extract on sensitized and non-sensitized cells. The response was used to determine the concentration at which cell viability was reduced by 50% (EC_{50}), this value was compared with a CTX3C standard [32]. Fish extracts were assayed at least in triplicate. Results were expressed as ng CTX3C per gram tissue equivalent (TE), a wet-weight measurement.

UHPLC-MS/MS analysis methods

UHPLC-MS/MS analyses were performed on a system consisting of an Agilent 1290 Infinity II UHPLC (Agilent, Waldbronn, Germany) connected to a Sciex QTrap 6500+ (Sciex, Darmstadt, Germany) as previously described in Spielmeyer, et al. [25]. In the case of the sodium adduct analysis, twenty different ion transitions (multiple reaction monitoring, MRM) were monitored to cover over 30 reported CTX congeners within one analytical run. Due to the high stability of the sodium adducts, the same *m/z* was selected both in quadrupole 1 and 3. For compound confirmation, product ions of the ammonium adducts were analyzed by monitoring four MRM transitions for each congener, with fragments

corresponding to the $[M + H]^+$, $[M + H\text{-}H_2O]^+$, $[M + H\text{-}2H_2O]^+$, and $[M + H\text{-}3H_2O]^+$ of the respective congener (details provided in Spielmeyer, et al. [25]).

Results And Discussion

Account of the outbreak

Fish sold to a processor

Five fishing vessels were listed on the statement of the certified catch certificate included in the RASFF report [29]. The size of three of the five vessels was either 26/6m or 26/4m (length/beam) while two vessels could not be identified based on the name of the ships provided on the catch certificate or using information available on 'global fishing watch' [34]. The three identifiable vessels sail under the flag of India and operate out of the Southwestern tip of India (i.e., FAO 51). The available fishing history of the three vessels occurred mainly within the exclusive economic zone of India, fishing between 70–76° W and 5.5–18° N. On May 8th 2017, the 5 fishing vessels unloaded a 'quantity' (i.e., 2995, 3000, 3000, 2950, and 2960 kgs, for vessels 1–5, respectively) of fish labeled as Lutjanus sp. to a processing plant in the port city of Kochi, located within the state of Kerala, India. The 'verified weight landed' mentioned on the original European Community Catch Certificate listed for 'Frozen Red Snapper Steak Slice 3 cm thickness 1/3 pieces per kg. 800grs bag X 10/Carton – 8kg. Lutjanus sp., for vessels 1–5 was 1414, 1407, 1407, 1384, and 1388 kgs, respectively.

Processor submitted samples for CTX analysis

The fish underwent final packaging at the processor and was given a lot number 629/2017-08 (also referred to as lot number 85205 – 2217 in the RASFF report). A 1.5 kg portion of the 7,000 kg packaged lot was subsampled by the Central Institute of Fisheries Technology (India Council of Agricultural Research) in Kerala, India for CTX analysis only. Between July 5th and 18th 2017, the testing facility performed the Mouse Bioassay on the subsample according to IOC Manuals and guides No. 33, CH.08 1995, UNESCO [35]. Accordingly, a test certificate (included in the RASFF report) was issued on July 18th 2017 regarding the sample submitted under the name 'Frozen Red Snapper Steak Slice (*Lutjanus bohar*)' and following the test results, part D of the document remarks "The samples tested for Ciguatera was found absent". The certificate goes on to state that "the results stated above relate only to the items tested", and no additional supporting documents or data were provided. The following day, July 19th 2017 the Export Inspection Council (Ministry of Commerce and Industry, Government of India) issued a "health certificate for imports of fishery products intended for human consumption" with reference number EIA/KOC/2017-18/02374 and the local competent authority on the document was listed as the Export Inspection Agency, Kochi.

Processor applied for export

A European Catch Certificate (Issued by the Competent Authority of India) was provided and a copy was included in the RASFF report, declaring the fishing vessels as being under the 'Marine Fishing (regulation) act of Kerala, India' (i.e., fish were harvested within the state of Kerala, including territorial waters along the coastline of the state) and fulfilling the requirements in Article 6 of EC regulation No. 1010/2009 regarding a system to prevent, deter and eliminate illegal, unreported and unregulated fishing. The included health certificate states the region of origin of the fish was FAO zone 51 (Western Indian Ocean). The date of shipment from the exporter was July 27th 2017. On August 1st 2017, the state authority validated the marine product for export to Antwerp, Belgium. The description of the shipped product was 7,000 kg of 'Frozen Red Snapper Steak Slice 3cm thickness 1/3 pieces per kg. 800grs bag X 10/Carton – 8kg. Lutjanus sp.'

Arrival and distribution of lots

The port of Antwerp, Belgium provided a bill of landing which listed: 'portion; 7000 kgs (875 cartons) of frozen red snapper steak. Temperature maintained at -21°C'. Sur Yon, France was listed as the destination for the imported product. No information was available regarding the product distribution until a bill of sale by the Wholesaler which sold '679 (quantity)' reported at 5928 kgs on January 29th 2019. From this point, a distribution list was provided with a product distribution beginning on February 6th 2019 and continuing until the April 24th 2020. A total of 341 cartons (each 8 kg) from lot 85205 – 2217 was distributed to 86 individual businesses in 63 postal codes, among nine EU countries and the United Kingdom. Distribution information regarding the other 534 cartons was not available. The original 'best before date' listed on the package was May 7th 2019. This date of expiration on this frozen product was extended until January 13th 2020 within the EU.

Outbreak report

The Netherlands Food and Consumer Product Safety Authority reported that five people within one household in the Netherlands consumed 'Red Snapper steak (*Lutjanus bohar*)' on May 14th 2020 (approximately 3 years after the fish were landed). A diagnosis of CP was provided by a healthcare professional, the consumers experienced gastro-enteritis after three hours and neurological symptoms were reportedly long-lasting (+ 21 days). Within the household, one original sealed bag (800 g tissue) was available for ciguatoxin analysis. This was not the package consumed, but was from the same batch and was purchased at the same time by the consumers. The sample was analyzed for CTXs by the Wageningen Food Safety Research Institute on July 14th 2020 using a two-tiered CTX analysis approach, consisting of a cell-based assay followed by LC-MS/MS (details provided below).

Traceback information

The fish product was exported from Thoppumpady which is located within the city limits of Koch, belonging to the state of Kerala, India (red square, Fig. 1). May 7th 2019 was listed as the product's original 'best before' date, this was extended to January 2020. The importer country was listed as the Netherlands and a wholesaler was identified from France. The fish lot was distributed to other countries

including Austria, Belgium, Finland, France, Germany, Italy, Luxembourg, Netherlands, Sweden, Switzerland, and the United Kingdom. The product was listed as destroyed after the passing of the best before date in Austria. In Finland, all product was sold before the notification. In Germany most product was not located, after being contacted some companies stated they do not carry the product in question, while some companies had been informed about the facts (e.g., outbreak, recall, and product information) by the company, while others stated they had no information about the facts of the case at the time of the investigation. In Italy, three kgs of the product remained in commerce and were scheduled for removal and disposal by an authorized company. In Luxembourg, all product was sold before the alert was registered. In the Netherlands, besides the original outbreak alert investigation, the remaining product was removed after the original expiration date (May 7th 2019). In Sweden all product was sold before the notification, however, a sign was displayed informing customers about whom to contact regarding the fish (no additional information was provided explaining if any callbacks occurred). In Switzerland the company listed as the recipient was no longer active at the time of the investigation, therefore tracing of the products was not possible. No additional information was provided from Belgium, France, or the United Kingdom.

DNA barcoding

DNA barcoding was performed to confirm the correct labeling of the species from the product lot implicated in the CP outbreak in the Netherlands. Therefore, the Cytb region was sequenced from two independent samples. The datasets generated and analysed during the current study are available in the NCBI repository, under the following accession number (ON759307, ON759308, ON759311, and ON759312). Sequence alignment confirmed that the analyzed samples were correctly labelled and belong to the species *L. bohar*. All sequenced samples had a base pair identity of 99%.

Toxin analysis by Neuro-2a cytotoxicity assay

Within this study, from the same lot as the CP outbreak, two sealed bags containing a total of seven pieces of fish were analysed at the BfR using the N2a cytotoxicity assay and all samples were determined to be positive for CTX-like toxicity (Table 1). All samples exhibited cytotoxicity only in the ouabain and veratridine pre-treated cells (OV+), confirming the sodium channel-specific mode of action resulting from the presence of CTX-like compounds contained in the sample extract (Fig. 2). The composite toxicity among all samples ranged from 0.79–5.39 ng CTX3C equivalent (eq.) per g wet tissue eq. (Table 1). Total toxin content among fish pieces ranged from 79.4–986 ng CTX3C eq. Based on the toxin content of each piece of fish, each bag recovered for testing contained a total of 1,965 and 1,690 ng CTX3C eq., respectively. Yasumoto [44] proposed a total CTX1B intake of 70 ng as a recommended limit for human health seafood consumption safety. Based on this recommendation these bags contained sufficient concentrations of CTXs to intoxicate multiple people. A bag of fish was also recovered at the home of the CP patients and was analyzed for CTXs using the N2a-cytotoxicity assay by the Food Safety Research Group at the University of Wageningen in the Netherlands. The Netherlands Food and Consumer Product Safety Authority reported that the samples were suspected to contain CTXs at levels above the US FDA

guidance limit of 0.01 µg CTX1B eq. per kg and provided an analytical report for further details. Therefore, the CP outbreak and subsequent testing of related material combined with the results of this study demonstrate that multiple bags of fish portions from this lot contained CTXs which are not allowed in fishery products sold in the EU.

Table 1
Sample description by weight and composite toxin quantification. The sample tissue extract's toxicity was determined from the effective concentration for reducing cell survival by 50% as compared with the standards CTX1B and CTX3C. Results are presented in ng of CTX equivalents per g of wet tissue as well as the total ng of CTX3C or CTX1B equivalent contained in each sample.

Sample									
	1	2	3	4	5	6	7	CTX1B	CTX3C
mg TE mL^{-1}	0.60 (± 0.04)	0.28 (± 0.03)	1.43 (± 0.03)	1.89 (± 0.13)	0.76 (± 0.08)	0.49 (± 0.19)	0.79 (± 0.21)	-	-
CTX3C eq.	2.49 (± 0.17)	5.39 (± 0.58)	1.05 (± 0.02)	0.79 (± 0.05)	1.98 (± 0.21)	3.05 (± 1.19)	1.90 (± 0.51)	-	1.50
CTX1B eq.	3.14 (± 0.21)	6.78 (± 0.73)	1.32 (± 0.03)	1.00 (± 0.07)	2.50 (± 0.26)	3.84 (± 1.50)	2.40 (± 0.65)	1.89	-
Weight (g)	305	183	133	100	112	280	323	-	-
Total toxin per fish piece									
ng CTX3C eq.	760 (± 50.6)	986 (± 106)	140 (± 2.94)	79.4 (± 5.48)	222 (± 23.3)	854 (± 333)	614 (± 166)		
ng CTX1B eq.	958 (± 63.8)	1,241 (± 132)	176 (± 3.70)	100 (± 6.90)	280 (± 29.4)	1,075 (± 419)	774 (± 209)		

Toxin identification by UHPLC-MS/MS

Sample extracts analyzed by LC-MS/MS revealed the presence of several putative CTX congeners such as 2,3,51-trihydroxyCTX3C, 2,3-dihydroxyCTX3C, 2-hydroxyCTX3C or M-*seco*-CTX3C (Fig. 3). Excluding 2,3,51-trihydroxyCTX3C, congeners generally consisted of two peaks eluting with retention times < 1 min

apart. The first peak is ascribed to the 49-epimer of the respective compound. Peak annotation was performed according to the *m/z*, the retention time (based on previously published elution profiles), and the fragmentation of the ammonium adducts (right column in Fig. 3) (additional details provided in Spielmeyer, et al. [25]).

The bag of fish recovered from the home of the CP patients was analyzed by the Food Safety Research Group at the University of Wageningen for brevetoxins using LC-MS/MS. Brevetoxins are ithyotoxic neurotoxins that can accumulate in fish and this test allowed them to exclude the possibility of a different marine biotoxin with a similar mode of action causing the observed effect in the N2a-MTT assay [45]. The results of the analysis showed the samples were negative for the presence of brevetoxins, however, the analysis report stated that CTXs could not be detected or confirmed, according to information provided in the analysis report included in the RASFF summary.

Catch region CP association

CP has a demonstrated association with geographic regions and species [6,9,13]. Samples of the species *L. bohar* were considered 'CTX positive' by the mouse bioassay from two geographic areas (Kerala and Karnataka) up to 400 km apart within the wider region of southwestern India, providing precedent for CTX-like toxicity in this species and region (Fig. 1) [42,43]. The southwestern region of India reported its first CP outbreak in 2015, with *L. bohar* being confirmed in subsequent CP incidences [40,41]. Specifically, in Mangalore (upper outbreak circle overlapping with an environmental sample on the border region of Karnataka and Kerala, Fig. 1), a major CP outbreak was reported in 2016 affecting 200 people. Seventy-five percent of the affected individuals were hospitalized with severe symptomology (neurological and gastrointestinal) and ten percent required extended hospitalization due to the severity of the cardiovascular symptoms experienced. Samples collected and tested from that large outbreak were investigated using the receptor binding assay and found to contain CTX-like activity equivalent to 1.10, 1.36, and 2.61 ng CTX3C eq. per g tissue for muscle, intestine, and liver tissue types, respectively [23]. LC-MS/MS investigations into the material suggested the Caribbean and Indian Ocean CTXs as the responsible ciguatoxin(s) [6,23]. The samples tested in this study were comparatively toxic and ranged from 0.79–5.39 ng CTX3C eq. per g wet tissue eq. Suggesting the removal of this CTX contaminated material from the commercial market by the responsible authority in Germany, following the RASFF alert, could be described as a preventative action, as several CP intoxications like those reported in the 2016 outbreak in India with similar toxin concentrations may have been avoided.

In 2017 a CP outbreak in the United Kingdom was reported and involved 1230 kg of frozen red snapper fillet which was a product from FAO area 51 (Indian Ocean). Subsequent testing on 24% of the lot revealed that all samples were positive for CTX-like toxicity by the N2a-assay and the samples contained CTXs with chromatographic peaks attributed to potential C/I-CTXs [46]. In contrast to I-CTXs, which remain structurally un-resolved and therefore complex to detect in outbreaks involving I-CTXs, no C/I-CTXs were detected in the samples from this study but rather several compounds in the CTX3C-group. The presence of CTX3C-group compounds may provide a CTX profile which could be further confirmed in other *L. bohar*

from the Indian Ocean region, particularly in events where CTX-like toxicity was observed and CTXs remain unresolved. CTX3C-group toxins have been described in *L. bohar* caught in the Pacific Ocean in FAO 61 [47] and 71 [20]. *L. bohar* was attributed to a CP outbreak in Germany in 2015 and described to contain 51-hydroxy CTX3C, the sample was purportedly a product of catch region FAO 51 (the western Indian Ocean) [18]. For another outbreak in Germany in 2012 involving *L. bohar* and *L. argentimaculatus* from FAO 57 (the eastern Indian Ocean) detection of CTX1B and 2,3-dihydroxyCTX3C were reported, however, no additional data was provided regarding which species contained the reported toxins [18]. *L. bohar* from the bank's fishery to the north of the Republic of Mauritius was reported to contain I-CTXs, this area is also part of FAO 51, but is located in the fishing territory's extreme southwestern portion [48]. The fishing vessels listed in this study were small (26 m length boats) and localized to the southwestern coastal region of India and therefore unlikely to have traveled over 4000 nautical miles round trip to fish in the Mauritius region. As of 2018, southwestern India has considered the existence of ciguatera as 'rare' but has nonetheless implemented monitoring fish for CTXs [49]. Southwestern India is a major marine fishing region, contributing to approximately 30% of India's total fisheries landings by weight (1.08 million tonnes) in 2019. Snappers as a category accounted for 10,246 tonnes of the 3.56 million tonnes of seafood landed throughout India in 2019 [50]. Therefore, the identification of CTXs in this species is of commercial importance and with potential CP ramifications for the wider regional fishery.

Follow-up actions from the EU

Follow-up actions and investigations by the competent authorities of the European Commission noted that the Export Inspection Council of India initiated actions against the establishment per the Executive instructions in force and the establishment was placed on "internal alert" on August 3rd 2020, stating that the export of red snapper to the EU was suspended until further order. The Export Inspection Council dispatched for a site examination observed that all red snapper (*Lutjanus bohar*) exported by the establishment weighed more than 5 kg and that available literature indicates an increased CP risk in fish over 2 kg. Accordingly, Oshiro, et al. [51] reported that 11.9% of *L. bohar* in Okinawa, Japan (region of highest CP rates in Japan) were CTX positive and no CTXs were detected among *L. bohar* weighing under 4 kg, providing precedent for the 5 kg weight restriction. The investigation concluded that the establishment's "own check system" of a species related to a hazard failed to identify and address the issue and failed to implement a raw material traceability system to help track the problem. Therefore, the Council concluded that because the establishment's traceback was insufficient and their product self-check did not work, these controls failed to prevent the distribution of fish containing CTXs to the destination. Currently, The European Commission has no responsibility for the export suspension of *L. bohar* from India and no additional information has been provided by the authorities in India regarding this outbreak (personal communication with the Directorate-General for Health and Food Safety March 29th, 2022).

CTX3C profile occurring in the Indian Ocean

This is the first complete description of multiple samples of *L. bohar* which were sourced from the Indian Ocean region and found to contain CTX3C-group compounds. Beyond the two mentions from the article by Friedemann [18] no descriptions of any CTX3C-group compounds originating in the Indian Ocean have been reported. Whether this is the first instance of a known CTX profile now reaching food web stability in a novel region, the FAO catch region was falsified (unlikely due to the certified catch record), or whether this profile has existed in the region as an undetermined CTX profile in seafood before this description requires further elucidation. The first description of CTX3C outside the Pacific occurred in the Atlantic, reported by Otero, et al. [52] followed by Silva, et al. [53], however, since these initial reports the CTX3C-group has not been described in the Atlantic region in any CTX analysis report. The *Gambierdiscus* complex including the species *G. polynesiensis* has been recently described in the northern Indian Ocean but remains undescribed for CTXs [54,55]. Cultures of *G. polynesiensis* from the Pacific Ocean have been demonstrated to produce CTX3C-group congeners (CTX3C/B, 2-hydroxyCTX3C, M-*seco*-CTX3C, CTX4A/B, and M-*seco*-CTX4A/B) [56–59]. Therefore, if this cosmopolitan species can produce the same suite of CTXs as those originating from the Pacific Ocean, then the CTX3C-group toxins identified in this study could originate from *G. polynesiensis*. Among the four groups of CTXs currently described only I-CTXs and the CTX3C-group have no specific regulation on toxin content guidance levels. Results presented here demonstrate that CTX3C-group congeners can be present at concentrations capable of causing CP without the presence of an additional CTX congener group and should be elevated to a CTX group of monitoring importance regarding suspected CP outbreaks.

Conclusion

The international seafood trade supplies products to consumers that are generally considered to be beneficial to society, but in rare cases the products distributed can present risks to human health. In this CP outbreak, the tracing back of CTX contaminated material resulted in the identification of a CTX3C-group with the potential to elucidate an attributable compound for the broad issue of CP outbreaks occurring in the southwestern Indian region. The seafood lot investigated herein was certified for export based on accurate catch records (adhering to international harvest laws) and up-to-date health certificates, permitting the large lot (7,000 kg) to be internationally disturbed among ten countries. The frozen product *L. bohar* was found to contain CTXs following an investigation of a reported CP outbreak. This species has been implicated in a mass CP outbreak in southwestern India (catch and export region) and is recognized as a CP risk species throughout much of its global native range. This frozen product posed a long-term CP risk, causing a CP outbreak 3 years post-harvest, and remained CTX positive by biological and analytical methods > 4 years, adhering to known CTX stability studies. CTX3C-group compounds are associated with CP outbreaks and toxic fish originating from the Pacific Ocean (e.g., FAO catch regions 61 [47], 71 [20], 77 [60,61] and 81 [62]). The identification of CTX3C-group compounds outside this range in FAO 51 may necessitate a re-investigation of the dogma of regional CTXs, particularly in seafood from the Indian Ocean. Furthermore, the region of export's local designation as a CTX free zone should be re-evaluated. Prior unresolved CP outbreaks in the region of southwestern India would benefit from a re-investigation of CTX suspected material for CTX3C-group toxins as described herein. The toxin content of

the fish exceeded all available CTX guidance values for human consumption. Therefore, this study serves as another example of CP prevention efforts based on product reclamation following a CP outbreak and for investigating remaining products for CTXs which are still on the market. In follow-up studies, regional investigations utilizing benthic surveys for a responsible *Gambierdiscus* spp. should be conducted to verify the algal source of CTXs and the investigation of seafood species with a small homerange to identify CTX trophic transfer pathways in the catch region. Identifying the CTX source and trophic transfer pathway will help inform resource managers to prevent future outbreaks of CP involving seafood from this region.

Declarations

Author contributions. CRL and AS designed the study and created the graphics. CRL, AS, VB, and DB, collected and analyzed the data. CRL wrote the original manuscript. CRL, AS, and OK edited the manuscript.

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Competing interests. None to declare

Data availability: All data generated or analysed during this study are included in this published article.

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Figures

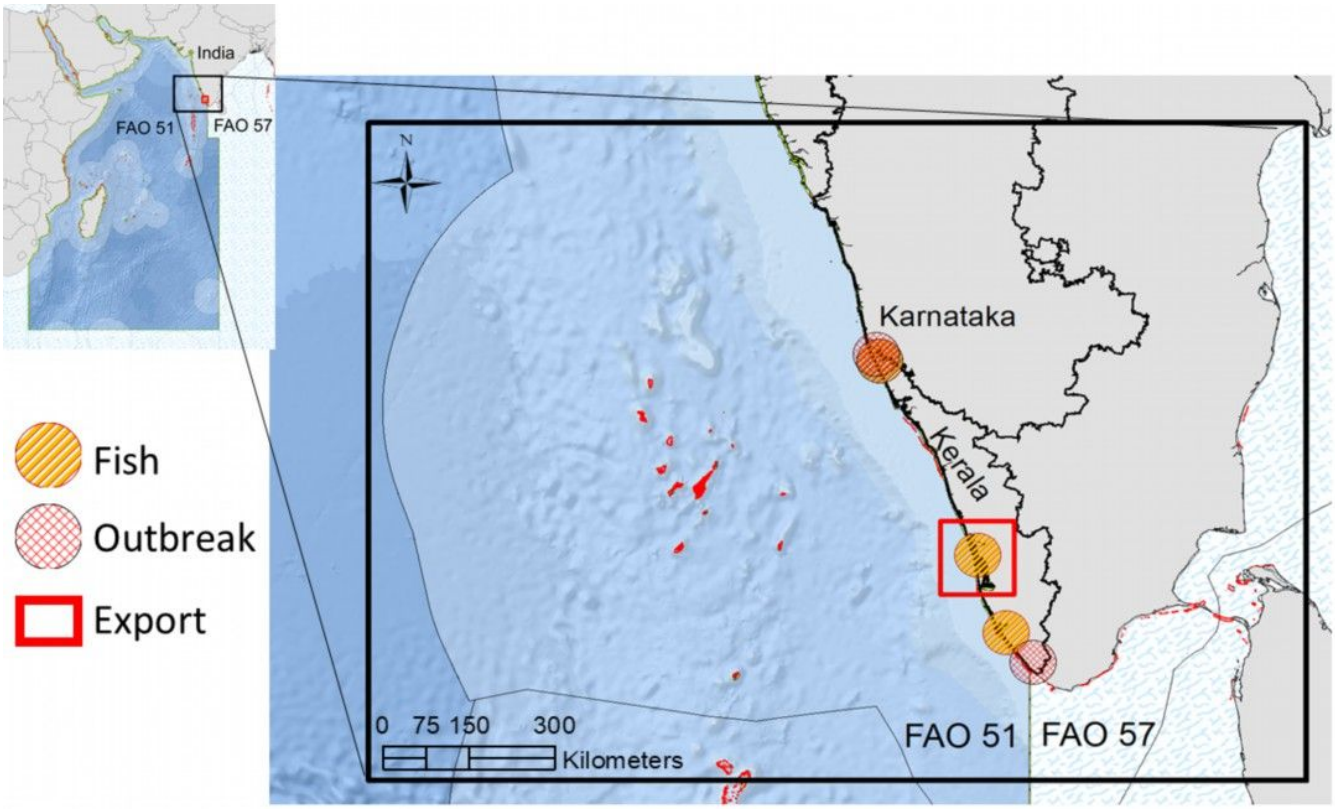

Figure 1

Map displaying the border extent of the Fish and Agricultural Organizations recognized fishing zone 51 [36], surrounding waters [37], exclusive economic zones (EEZ) of neighboring countries (the thin gray line is the border, lighter blue color is inside the EEZ) [38], known coral reefs indicated by red marks [39], and symbols representing locations of interest. The world map (upper left) contains a black square indicating the regional area depicted in the main map figure. Main map figure shows southwest coastal India with a

focus on the states of Karnataka and Kerala (outlined). Circles with orange diagonal lines represent areas where L. bohar were tested and found to be CTX-positive by the mouse bioassay [40-43]. Circles with hash marks in red were from CP outbreaks. Red square indicates the location of export Thoppumpady, Kerala, India, for lot number 629/2017-08 which was implicated in a ciguatera outbreak reported on May 14th 2020.

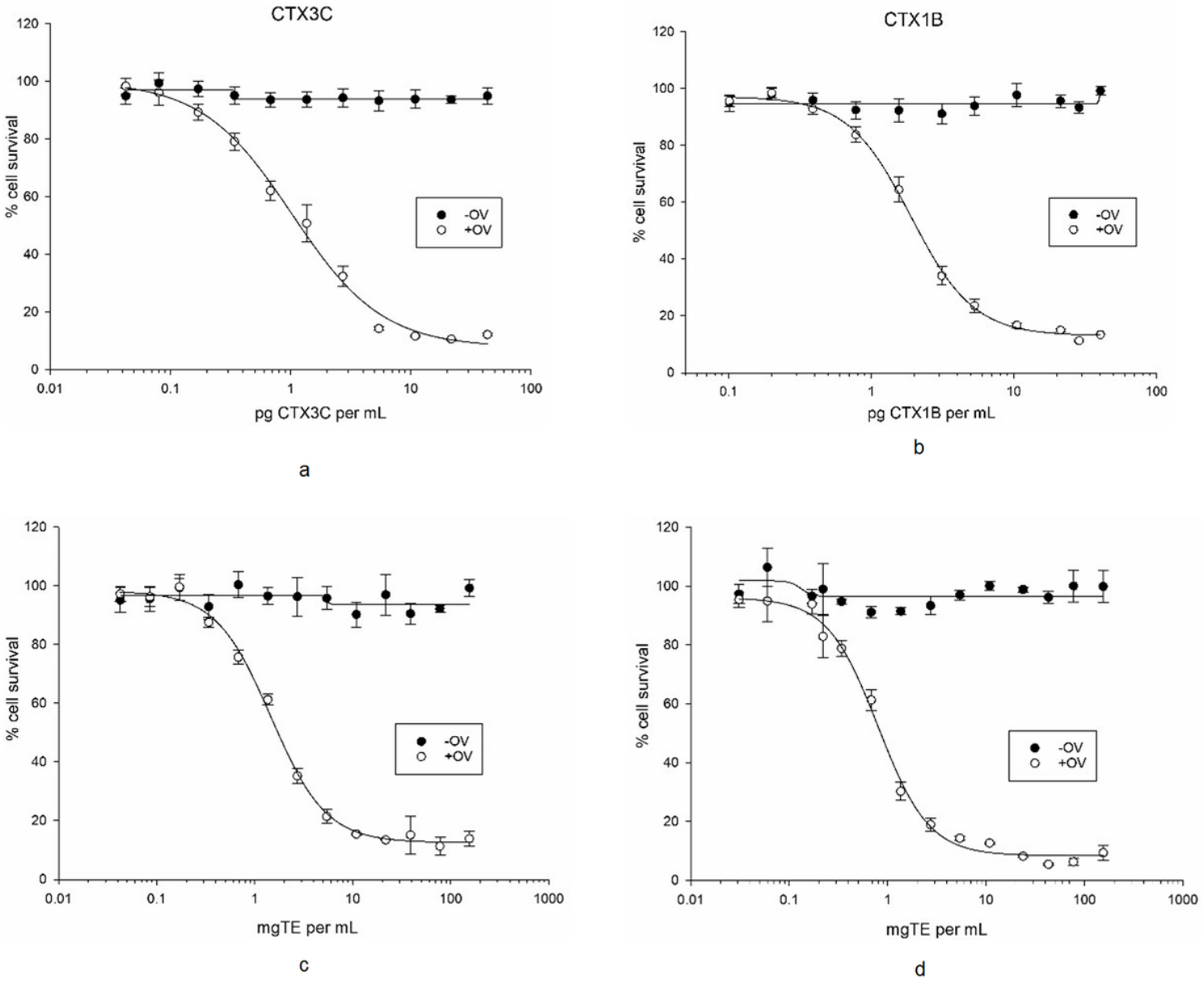

Figure 2

Combined concentration-response curves of N2a cells without (-) the addition of ouabain (O) and veratridine (V) (–OV, solid symbols) and with the addition of OV (+OV, open symbols), when exposed to various concentrations of either a standard of (a) CTX3C, (b) CTX1B, or (c,d) semi-purified extracts of fish in tissue equivalents (TE) (c = sample #3 and d = #7). OV-LS N2a cells were exposed to 0.22/0.022 mM

O/V. Error bars represent the standard deviation from all independent 96-well plate analyses performed for each sample (minimum 3 independent assays, each assay includes 3 replicate points).

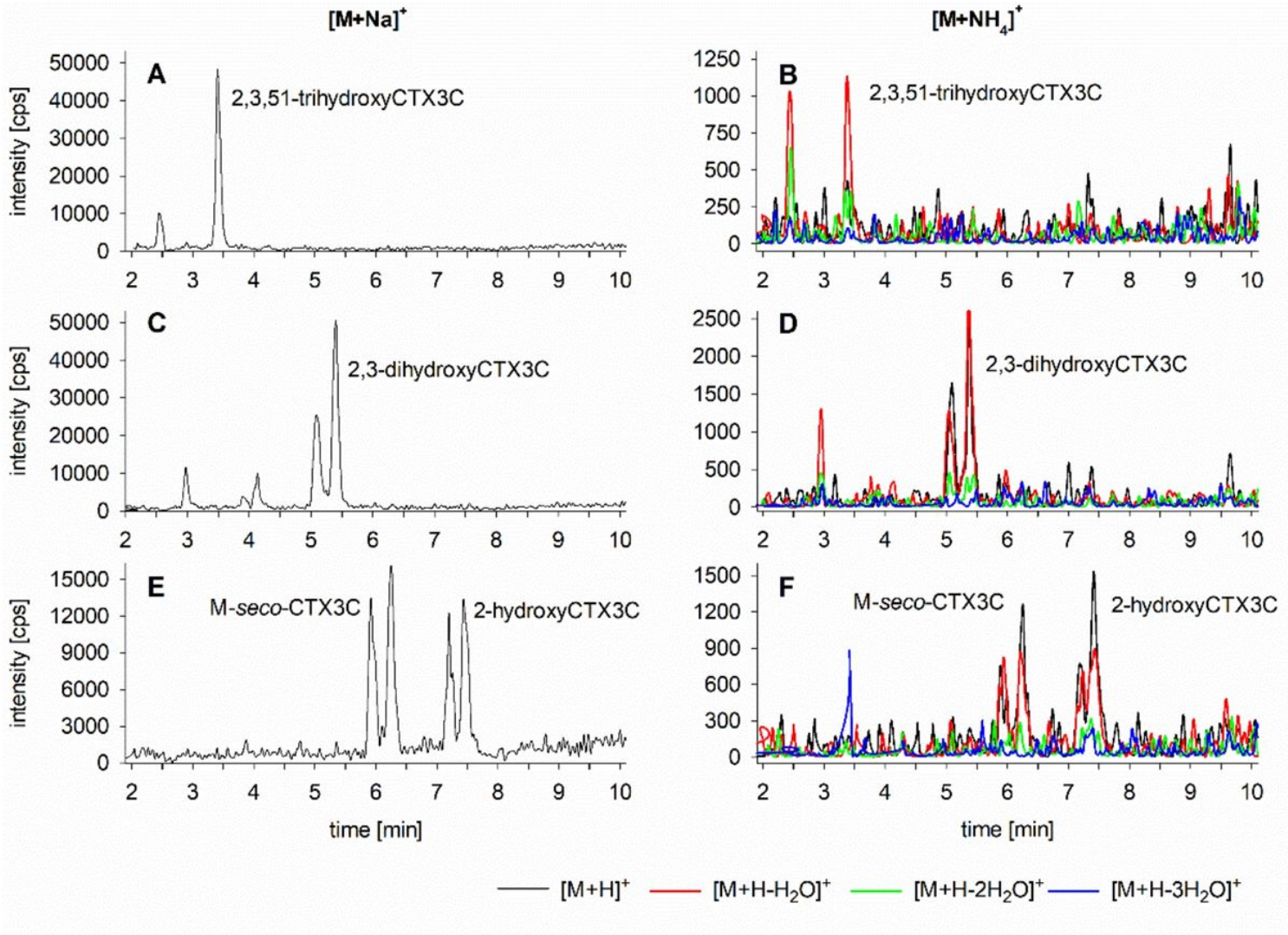

Figure 3

LC-MS/MS chromatograms obtained for sample 7; graphs show the extracted ion chromatograms of the respective m/z for 2,3,51-trihydroxyCTX3C, 2,3-dihydroxyCTX3C, M-seco-CTX3C, and 2-hydroxyCTX3C for the analysis of the sodium adducts ($[M+Na]^+$, left column), and the analysis of the fragments of the ammonium adducts ($[M+NH_4]^+$, right column). The color code for the right column is provided in the figure; details concerning the peak annotation are provided in Spielmeyer, et al. [25].