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Ciguatera poisoning in Europe: A traceback to Indian Ocean sourced snapper fish (*Lutjanus bohar*)

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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. The southwestern coast of India is a regional source of seafood attributed to isolated and mass outbreaks of CP since 2015, both locally and exported globally. Samples of frozen snapper product (*Lutjanus bohar*) described herein, were part of a 7000 kg international shipment into the European Union from southwest India and implicated in a CP outbreak in the Netherlands. DNA barcoding confirmed the species as *Lutjanus bohar* with a base pair identity of 99%. LC-MS/MS and HRMS analyses describe CTX-3C-group compounds with an *in vitro* Neuro-2a (cell-based) cytotoxicity MTT-assay based toxicity range of 0.79–5.39 ng CTX-3C equivalent (eq.) per g wet tissue eq. This CP traceback includes an investigation and description of the production chain distribution, catch region, outbreak, toxin-group, and follow-up actions for the seafood products associated with the outbreak. Together this in-depth traceback investigation provides an account of the CP outbreak from harvest to consumption for a region of coastal India with a sizable seafood production industry but with limited CP data.

1. 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 from CP annually, with symptoms that may include gastrointestinal, neurological, and cardiovascular symptomology (as reviewed by Friedman et al. (2017)). Under- and de-centralized CP case reporting, undiagnosed cases, and difficulties in toxin identification are commonly cited problems, restricting the accurate accounting of CP (Bilbao-Sieyro et al., 2019; Friedman et al., 2017; Skinner et al., 2011; Tester et al., 2010).

CTXs are potent neurotoxins produced by microalgae in the genera *Gambierdiscus* and *Fukuyoa*. Animals that ingest these microalgae can assimilate CTXs through their diet (i.e., biomagnification), and following their ingestion, the toxins are incorporated throughout the consumer's body. Marine animals with CTXs can be found in various food webs and habitats within tropical, subtropical, and some temperate zones, as reviewed by various authors (Chinain, Gatti, Darius, et al., 2020; FAO and WHO, 2020; Tester et al., 2018). CTXs are organoleptically undetectable in food products and resistant to cooking, freezing, or general food preparation techniques (Dickey & Plakas, 2010). A total CTX-1B intake of 70 ng has been suggested as a safety limit for human health consumption (Yasumoto, 2005). CTX detection at human health-relevant concentrations (e.g., 0.01 μg CTX-1B equivalents (eq.) per kilogram of tissue as recognized by the US Food and Drug Administration (US FDA) (Dickey & Plakas, 2010; U.S. Food Drug Administration, 2020)) 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), cellular-based assays, or enzyme-linked immunosorbent assay (ELISA) (Tsumuraya et al., 2014; Tsumuraya & Hirama, 2019) operated by trained personnel.

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Despite these recognized detection, prevention, and epidemiological complexities, guidance for products containing CTXs exist to try and safeguard consumers. Within the European Union's (EU) jurisdiction, products containing CTXs must not be placed on the market (Regulation (EC) No 627/2019; Regulation (EC) No 853/2004; EFSA, 2010). In endemic and non-endemic regions, CP management efforts are based on the local authority's historic knowledge of, or association with, CP, generally relying on harvest restrictions by location, species, sizes (weight or length), or some combination thereof (Sydney Fish Market, 2015; Loeffler et al., 2018; Sanchez-Henao et al., 2019; U.S. Food Drug Administration, 2020; Canals et al., 2021; Loeffler, Abraham, et al., 2022). In the EU according to Article 4 of 2000/104/EC (Regulation (EC) No 104/2000, 2000), the label of any fishery products on sale must contain the commercial name of the species, the production method (capture method), and the harvest 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). Mislabeling species of snappers is recognized as a global value chain problem and some commonly mislabelled species can have associated CP health risks (Friedman et al., 2017; Kusche & Hanel, 2021). CTX-contaminated products have unwittingly been distributed globally by the international seafood trade as evidenced by the reoccurrence of imported products resulting in CP outbreaks (Friedemann, 2019; Loeffler, Spielmeyer, et al., 2022; Varela Martínez et al., 2021).

Over 30 CTX analogues have been identified to date (FAO and WHO, 2020), however, only two CTX standard substances are commercially available, CTX-1B and CTX-3C (both are from the group associated with the Pacific region). Four structural groups of CTXs have been described among three Oceanic basins: CTX-4A and CTX-3C 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. (2021) 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 (Diogène et al., 2017; Karunasagar et al., 2018), emphasizing that closing the CTX knowledge gaps remains critical for this region.

Generally, CTXs are food contaminants without validated detection methods (EFSA, 2010; FAO and WHO, 2020), 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 CTX-1B eq. per kg). These problems can be further exacerbated when products are improperly labeled (either by species or catch regions) (Kusche & Hanel, 2021; Loeffler, Spielmeyer, et al., 2022), as controls that rely on an accurate species or regional history of CP can be bypassed. Furthermore, analytical assumptions for targeting regionally associated CTX compound(s) (i.e., C-CTX-1 in the Caribbean or CTX-1B in the Pacific) can miss unknown or regionally novel CTXs if products are mislabelled or in the case of regionally emergent CTXs.

Only a handful of CP cases have a clinical diagnosis and even fewer 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 ideally required, including a medical diagnosis, toxicological investigation of the meal remnant for the attributable CTX analog with an ascribed toxin concentration, species authentication for the meal remnant, and traceback to the harvest location. This information is essential for identifying relevant factors to identify toxin production areas and address human health and monitoring questions.

Historically, CP symptomology and CTX molecular descriptions were associated with an Ocean basin, as reviewed by Friedman et al. (2017); FAO and WHO (2020). In the Pacific, regional symptoms are predominately 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(s) and have on occasion reported unique symptoms (i.e., hallucinations) and mental depression (Habermehl et al., 1994; Lewis, 2001; Quod & Turquet, 1996). Herein we provide comprehensive details on an internationally traded lot of *Lutjanus bohar* originating from the Indian Ocean that was responsible for an outbreak of CP in the Netherlands in 2020. This description includes an account of the outbreak, traceback to the harvest region, and CTX analysis results based on available portions of the corresponding seafood batch. Coastal India currently suffers from a paucity of available data regarding CP descriptions and confirmed CTXs (Habibi et al., 2021). The description 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, as reviewed by Habibi et al. (2021).

2. Methods

2.1. Material collection and reagents

According to the Rapid Alert System for Food and Feed (RASFF) notification number 430888, with reference number 2020.2254 (Commission, 2020), a food poisoning alert notification regarding a serious human health risk for CP was sent on May 29th, 2020 following a documented CP outbreak. 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.

According to the product distribution list, two sealed bags (packaged on May 8th, 2017 with a best-by date of May 7th, 2019) from the same lot were collected from individual businesses on June 2nd, 2020 in Bonn and Mönchengladbach, Germany. 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. The bags contained seven portions of fish (samples 1–4 from bag one and samples 4–7 from bag two) and were transferred frozen and in good condition to the German Federal Institute for Risk Assessment (BfR) for CTX analysis.

All cell line consumables and working conditions were performed following Loeffler et al. (2021) and Loeffler, Spielmeyer, et al. (2022). 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.

Methanol standard solutions of CTX-1B (4 μg mL^{-1}), 52-*epi*-54-deoxyCTX-1B (i.e., P-CTX-2, 1 μg mL^{-1}), and 54-deoxyCTX-1B (P-CTX-3, 2 μg mL^{-1}) were purchased from Professor R. J. Lewis (The Queensland University, Australia, prepared November 2005). CTX-3C (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.

2.2. 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 (DIN EN ISO 21571:2013-08, 2013). DNA barcoding was performed according to DIN CEN/TS 17303:2019. Cytochrome *b* (Cytb) barcoding region was amplified with primers L14735 (5′-AAAAACCACCGTTGTTATTCAACTA-3′) and H15149ad (5′-GCICCTCARAATGAYATTTGTCCTCA-3′) in 25 μL reaction tubes in a Master cycler 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 the Basic Local Alignment Search Tool (BLAST).

2.3. 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 for the N2a-MTT assay by Dickey (2008) and Spielmeyer et al. (2021) for LC-MS/MS and high resolution (HR) 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 cartridge. 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. Details concerning cell based assay parameters and tests are provided in the Supplemental Material.

For LC-MS/MS and HRMS 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 first by reversed-phase SPE. The obtained eluate was further purified by 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. Both fractions were utilized for sample analysis, with the filtrate containing parts of CTX-3C and the eluate containing CTX-3C in addition to more polar (e.g., 49-*epi*-CTX-3C) congeners.

2.4. LC-MS/MS and HRMS analysis

LC-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. (2021). HR MS analyses were performed on a system consisting of an Agilent 1290 Infinity II UHPLC connected to a Sciex TripleTOF 6600+. Details concerning UHPLC and MS parameters are provided in the Supplemental Material (Supplementary Tables 1 and 2).

3. Results and discussion

3.1. DNA barcoding

DNA barcoding was performed to confirm the correct labeling of the species (*Lutjanus bohar*) from the product lot implicated in the CP outbreak in the Netherlands. Therefore, the cytochrome *b* gene (Cytb) region was sequenced from two independent samples. The datasets generated and analyzed during the current study are available in the supplementary information file and the NCBI repository, under the following accession number: ON759307 (https://ncbi.nlm.nih.gov/nuccore/ON759307), ON759308 (https://ncbi.nlm.nih.gov/nuccore/ON759308), ON759311 (https://ncbi.nlm.nih.gov/nuccore/ON759311), and ON759312 (https://ncbi.nlm.nih.gov/nuccore/ON759312). Sequence alignment confirmed that the analyzed samples were correctly labeled and belonged to the species *L. bohar* as found on NCBI (Miller & Cribb, 2007). All sequenced samples had a base pair identity of 99%. Additionally, visual comparisons of the sequences obtained from the *L. bohar* herein, and sequence analysis with other Lutjanus species, showed only 91% similarity. Species identification based on cytochrome oxidase I gene (COI) and Cytb are the most common genetic loci used. However, taxonomically authenticated genetic reference material for conducting food authentication studies is a globally recognized limitation for DNA sequence analyses. Often authentic reference material is lacking altogether, questionable in authenticity, or not available on public data bases (Kusche & Hanel, 2021; Naaum & Hanner, 2016).

3.2. Analysis of sample extracts

Extracts were analyzed by both cellular-based assay on neuroblastoma (N2a-assay) and LC-MS/MS. All extracts showed a CTX-like toxicity in the cell based assay. Details for each sample are provided in the Supplementary Material (Supplementary Table 3, Supplementary Fig. 1).

Sample extracts analyzed by LC-MS/MS revealed the presence of several putative CTX congeners such as 2,3,51-trihydroxyCTX-3C, 2,3-dihydroxyCTX-3C, 2-hydroxyCTX-3C or M-*seco*-CTX-3C. Excluding 2,3,51-trihydroxyCTX-3C, analogues 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* (Supplementary Table 1), the retention time (based on previously published elution profiles by Yasumoto et al. (2000); Yogi et al. (2011), details provided in the Supplementary Table 2), and the fragmentation of the ammonium adducts (observation of at least two of the four recorded fragments) (right column in Fig. 1) (additional details provided in Spielmeyer et al. (2021)). It was further supported by HRMS analyses (Supplementary Table 4).

3.3. Harvesting, distribution, outbreak account, and traceback

3.3.1. Fish harvested and processed in India

Five fishing vessels were listed on the statement of the 'certified catch certificate' which was included in the RASFF report (European Union, 2020). Two vessels were unidentifiable, the other three sail under the flag of India and operate out of the Southwestern tip of India (i.e., FAO 51), indicating the territorial waters in which the vessels are permitted operate. These boats were either 26/6m or 26/4m (length/beam), based on information available in the catch certificate or using information available on 'global fishing watch' (Global Fishing Watch, 2022). The available fishing history of the three vessels indicated operating mainly between 70 and 76° W and 5.5–18° N (part of the exclusive economic zone of India). On May 8th, 2017, the five fishing vessels sold 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. 800 gr bag X 10/Carton – 8 kg. Lutjanus sp.,' for vessels 1–5 was 1414, 1407, 1407, 1384, and 1388 kg, respectively.

After the fish were landed, they underwent final processing and packaging, and were given a traceable lot number 629/2017–08 (also referred to as lot number 85205–2217 in the RASFF report). From this packaged product a 1.5 kg portion of the 7000 kg lot was subsampled for CTX testing by the Central Institute of Fisheries Technology (India Council of Agricultural Research) in Kerala, India. According to the Test Certificate provided (a copy was included in the RASFF report), between July 5th and 18th, 2017, the subsample was tested by the Mouse Bioassay according to IOC Manuals and Guides No. 33, CH.08 1995, UNESCO (Hallegraeff et al., 1995). On July 18th, 2017 the sample tested was deemed 'negative' with a further remark that 'the samples tested for

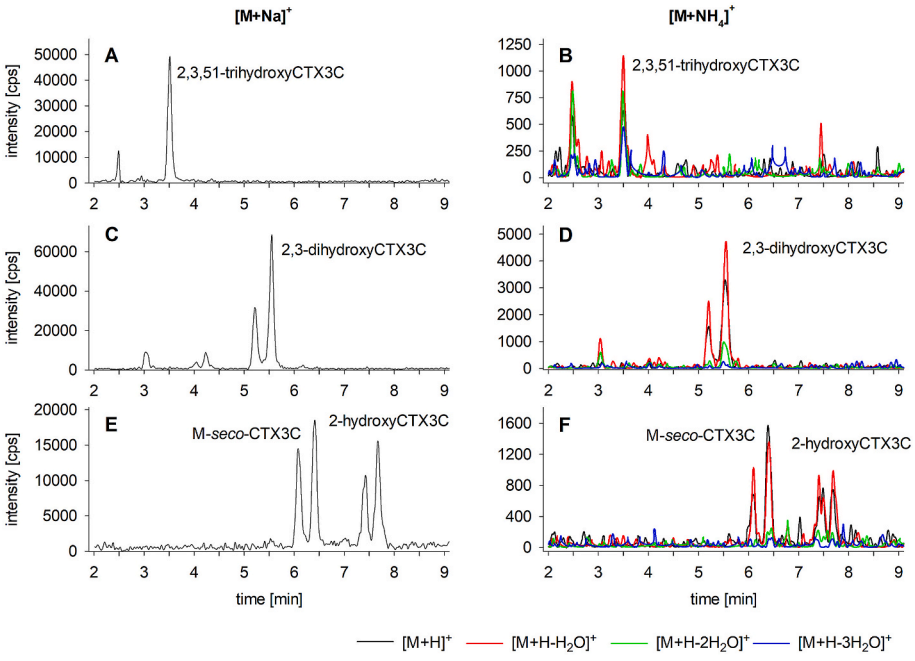

Fig. 1. LC-MS/MS chromatograms obtained for sample 7; graphs show the extracted ion chromatograms of the respective *m/z* for 2,3,51-trihydroxyCTX-3C, 2,3-dihydroxyCTX-3C, M-*seco*-CTX-3C, and 2-hydroxyCTX-3C for the analysis of the sodium adducts ($[M+Na]^+$, left column), and the analysis of the fragments of the ammonium adducts ($[M + NH_4]^+$, right column) (details about the *m/z* can be found in Supplementary Table 1). The color code for the right column is provided in the figure; details concerning the peak annotation are provided in Spielmeyer et al. (2021).

Ciguatera was found absent' with an additional note that 'the results stated above relate only to the items tested'. No additional supporting documents or data were provided. The following day, 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.

A European Catch Certificate, Issued by the Competent Authority of India, was provided (a copy was included in the RASFF report (European Union, 2020)). This certified that the fishing vessels were compliant with 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). The certification fulfills the requirements in Article 6 of EC regulation No. 1010/2009 regarding a system to prevent, deter and eliminate illegal, unreported, and unregulated fishing which is an important traceback point for CP. The health certificate states the fish were from FAO zone 51 (Western Indian Ocean). On August 1st, 2017, the state authority validated the marine product for export to Antwerp, Belgium.

3.3.2. *Arrival and distribution of lots in europe*

The port of Antwerp, Belgium provided a bill of landing which listed: 'portion; 7000 kg (875 cartons) of frozen red snapper steak. Temperature maintained at −21 °C' indicating the product was properly handled during transport. While the product entered via Antwerp, its final destination was Sur Yon, France. From here, no information was available regarding the product distribution until two years later on January 29th, 2019 when the Wholesaler reported a sale of 5928 kg. From this timepoint, a distribution list was provided with a product distribution beginning on February 6th, 2019, and continuing until April 24th, 2020. A total of 341 cartons (each 8 kg) from lot 85205–2217 were distributed to 86 individual businesses in 63 postal codes, among nine EU countries and the United Kingdom indicating this product was widely distributed. Distribution information regarding the other 534 cartons were not available (i.e., the whereabouts, distribution, or impact of this product were unknown).

3.3.3. *CP 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 3 h and neurological symptoms were reportedly long-lasting (+21 days). Within the household, one original sealed bag (800 g tissue) was available for CTX 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 cellular-based assay (N2a-assay) followed by LC-MS/MS. Their analysis report was included in the RASFF summary (European Union, 2020) and stated that CTXs could not be detected or confirmed by LC-MS/MS. However, the report stated that the samples were toxic by the N2a-assay, at levels above the US FDA guidance limit of 0.01 μg CTX1B eq. per kg. Fishery products containing CTXs shall not be placed on the EU market and based on these results multiple bags of fish from this lot have been demonstrated to contain CTXs (e.g., a documented CP outbreak, a CTX-like positive result in the Netherlands, and the results of this study). Because brevetoxins are ichthyotoxic neurotoxins that can accumulate in fish and produce a similar mode of action which can cause similar effects in the N2a-MTT assay (Naar et al., 2007), the samples were also investigated for the presence of brevetoxins by LC-MS/MS and were negative.

3.3.4. *Traceback information*

The fish product was exported from Thoppumpady, India (red square, Fig. 2), imported to the Netherlands, and distributed to other European countries (Austria, Belgium, Finland, France, Germany, Italy, Luxembourg, Netherlands, Sweden, Switzerland, and the United Kingdom). Among these countries, the remaining product was only available in Germany. In Austria and the Netherlands (besides the outbreak) the product was removed or destroyed after the passing of the best-before date. In Finland, Sweden, and Luxembourg, all product was sold before the notification. In Italy, three kg of the product remained in commerce and was scheduled for removal and disposal by an authorized company. In Switzerland, the company listed as the recipient was no longer active at the time of the investigation, therefore tracing the products was not possible. No additional information was provided from Belgium, France, or the United Kingdom.

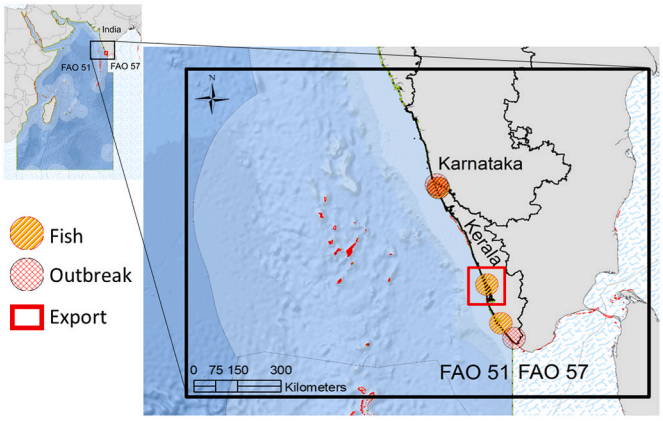

Fig. 2. Regional map of the product export location, with regionally reported CP descriptions of local outbreaks and environmental samples generated from published literature. The main map figure shows southwest coastal India with a focus on the states of Karnataka and Kerala (outlined). Circles with orange diagonal lines represent locations where reports of *L. bohar* were tested and found to be CTX-positive by the mouse bioassay based on the following references (Rajeish et al., 2016; Rajisha et al., 2017a, 2017b; Rajisha & Kumar, 2018). Circles with hash marks in red were from reported CP outbreaks. The red square indicates the location of export Thoppumpady, Kerala, India, for lot number 629/2017–08 which was implicated in a CP outbreak reported on May 14th, 2020. Maps created using ArcGIS (version 10.2) (Esri Inc., 2022a) with the following layers: Ocean basemap (Esri Inc., 2022b) displaying the border extent of the Fish and Agricultural Organizations recognized fishing zone 51 (FAO, 2014), surrounding waters (Flanders Marine Institute, 2021), exclusive economic zones (EEZ) of neighboring countries (the thin gray line is the border, lighter blue color is inside the EEZ) ((VLIZ), 2014), known coral reefs indicated by red marks (Institute for Marine Remote Sensing, 2011), 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.

3.3.5. Follow-up actions from the EU

The competent authorities of the European Commission noted that 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. An Export Inspection Council of India conducting a site examination observed that all red snapper (*L. bohar*) exported by the establishment weighed more than 5 kg. The fish weight relationship has precident for CP, in a study by Oshiro et al. (2010) of *L. bohar* in Okinawa (the region of highest CP rates in Japan), it was reported that 11.9% of *L. bohar* tested were CTX positive, that CP risk increased in fish over 4 kg, and no CTXs were detected in specimens weighing under 4 kg. However, conclusive CP studies on *L. bohar* in the southwestern coastal Indian region are not available. The European Commission 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. The European Commission is responsible for imports into the EU market and therefore has no responsibility for any export suspension of *L. bohar* from India, and furthermore, the authorities in India have provided no additional information regarding this outbreak (personal communication with the Directorate-General for Health and Food Safety March 29th, 2022).

3.3.6. CP association by catch region, species, and toxin profile

Southwestern India is a major fishing region, contributing 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 (CMFRI, 2020). As of 2018, the existence of CP in this region was considered 'rare', but *L. bohar* has been implicated in CP outbreaks since 2015 (Rajisha et al., 2018) (Fig. 2). CP has a demonstrated association with geographic regions and species (Sydney Fish Market, 2015; FAO and WHO, 2020; U.S. Food Drug Administration, 2020). *L. bohar* harvested from Kerala and Karnataka in southwestern India were tested using the mouse bioassay and deemed positive for CTXs, indicating this species' potential for CP in the region (Fig. 2) (Rajisha, Kishore, Panda, Ravishankar, & Kumar, 2017; Rajisha & Kumar, 2018). The first report of a CP outbreak in southwestern India was in 2015 and *L. bohar* was since confirmed in subsequent CP incidences (Rajeish et al., 2016; Rajisha, Kishore, Panda, Harikrishnan, et al., 2017). In 2016 a major outbreak was reported, affecting 200 people in Mangalore (upper outbreak circle overlapping with an environmental sample on the border region of Karnataka and Kerala, Fig. 2). 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 CTX-3C eq. per g tissue for muscle, intestine, and liver tissue types, respectively (Karunasagar et al., 2018). LC-MS/MS investigations into the material suggested the Caribbean and Indian Ocean CTXs (i.e., C-CTXs and I-CTXs, respectively) as the responsible ciguatoxin(s) (FAO and WHO, 2020; Karunasagar et al., 2018). 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 zone 51. Samples tested from the lot were positive for CTX-like toxicity by the N2a-assay and described to contain CTXs with chromatographic peaks attributed to potential C-CTX or I-CTX group congeners (Varriale, 2021, p. 408).

Structures of the I-CTXs remain unresolved and therefore these congeners are complex to detect in outbreaks involving I-CTXs. C-CTX-1 and I-CTX-1 possess the same *m/z* and similar structures are suspected for these compounds based on their chromatographic elution (Diogène et al., 2017; Hamilton, Hurbungs, Jones, & Lewis, 2002). The respective *m/z* ion transitions for C-CTXs and I-CTXs reported in the literature were included in the method (see also Supplementary Table 1), but no peaks were detected in the samples from this study. *L. bohar* containing I-CTXs have only been reported from the Republic of Mauritius (Hamilton, Hurbungs, Vernoux, et al., 2002). While this area is also part of FAO 51, it is located in the extreme southwest portion of the fishing zone, and based on the available fishing vessel catch data and certification, it is unlikely the fish in this outbreak originated from that region.

However, several compounds in the CTX-3C-group were detected. Therefore, the presence of CTX-3C-group compounds may provide a CTX profile that 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, such as the mass outbreak from 2016. These compounds are historically associated with the Pacific Ocean (e.g., FAO catch regions 61 (Yogi et al., 2011), 71 (Loeffler, Spielmeyer, et al., 2022), 77 (Oshiro et al., 2021; Yogi et al., 2014), and 81 (Chinain, Gatti, Ung, et al., 2020)). How and why this Pacific-associated CTX profile was found in seafood from the Indian Ocean, requires further elucidation. CTX-3C-group toxins (51-hydroxyCTX-3C and 2,3-dihydroxyCTX-3C) in *L. bohar* from the Indian Ocean (FAO 51 and 57) have been described, but remain unconfirmed in those studies (Friedemann, 2019). The first description of CTX-3C outside the Pacific occurred in the Atlantic, reported by Otero et al. (2010) followed by Silva et al. (2015), however, since these initial reports the CTX-3C-group has not been described in detail from the Atlantic region. The *Gambierdiscus* complex including the species *G. polynesiensis* has been recently described in the northern Indian Ocean, but remains undescribed for CTXs from this area (Munir et al., 2011; Saburova et al., 2013). Cultures of *G. polynesiensis* from the Pacific Ocean have been demonstrated to produce CTX-3C-group congeners (CTX-3C/B,

2-hydroxyCTX-3C, M-*seco*-CTX-3C, CTX-4A/B, and M-*seco*-CTX-4A/B) (Chinain et al., 2010; Longo et al., 2019, 2020; Sibat et al., 2018). Therefore, if this cosmopolitan species can produce the same suite of CTXs in the Indian Ocean as *G. polynesiensis* originating from the Pacific Ocean, then the CTX-3C-group toxins identified in this study could originate from *G. polynesiensis*.

The samples in this study ranged from 0.79 to 5.39 ng CTX-3C eq. per g wet tissue eq. (Supplementary Table 3 and Supplementary Fig. 1). Because the range of toxicity exceeded the guidance levels for CTXs, 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 which avoided potential CP intoxications. Among the four groups of CTXs currently described, only I-CTXs and the CTX-3C-group have no specific guidelines on toxin content or guidance levels (Yasumoto, 2005; U.S. Food Drug Administration, 2020). Results presented here demonstrate that CTX-3C-group analogues 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. CTX-3C-group in FAO 51 may necessitate a re-investigation of the dogma of regional CTXs, particularly in seafood from the Indian Ocean, and the region of export's local designation as a CTX-free zone should be re-evaluated.

4. 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. The frozen seafood lot (7000 kg) of *L. bohar* from India implicated in the CP outbreak investigated herein was certified for international distribution (eventually reaching ten countries). However, due to a reported CP outbreak on May 14th, 2020, a traceback investigation was conducted, and several CTX-3C-group analogues in consumer packages from the responsible lot were described. Prior unresolved CP outbreaks in (or from) the region of southwestern India (catch and export region) could contain CTX-3C-group congeners. Frozen CTX contaiminated product can pose a long-term CP risk, described herein to remain CTX positive by biological and analytical methods >4 years post-harvest. The toxin content of the remaining outbreak related material exceeded all available CTX guidance values for human consumption. Therefore, this study serves as another example of the importance of identifying the production steps from harvest to consumption for CP outbreaks, the value of prevention efforts based on investigating the remaining products on the market for CTXs, and confirming *L. bohar*'s designation as a CP risk species. In follow-up studies, regional investigations utilizing benthic surveys for responsible *Gambierdiscus* spp. should be conducted to verify the algal source(s) of CTXs and the investigation of seafood species with a small home range to identify CTX trophic transfer pathways in the catch region. Identifying the CTX source(s) and trophic transfer pathways will help inform resource managers on how to prevent future outbreaks of CP involving seafood from this region.

CRediT authorship contribution statement

Christopher R. Loeffler: Writing – review & editing, Formal analysis, designed the study and created the graphics, collected and analyzed the data, wrote the original manuscript, edited the manuscript. **Astrid Spielmeyer:** Writing – review & editing, Formal analysis, designed the study and created the graphics, collected and analyzed the data, edited the manuscript. **Vincent Blaschke:** Formal analysis, collected and analyzed the data. **Dorina Bodi:** Formal analysis, collected and analyzed the data. **Oliver Kappenstein:** Writing – review & editing, edited the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2023.109799.

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