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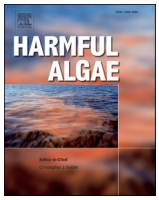

# Toxicity and toxin profile of La Réunion (Indian Ocean) fish containing CTX-like compounds

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ABSTRACT

Several outbreaks of ciguatera poisoning have been recorded in La Réunion (SW Indian Ocean) since 1986. Toxicities and toxin profiles of fish from the Indian Ocean are poorly documented. In this work, a multidisciplinary study on the toxicity of fish from the Indian Ocean by neuro-2a cell-based assay was assessed, and the CTX-like compounds were evaluated by a magnetic bead-based immunoassay. The toxin profile is described by liquid chromatography coupled to tandem mass spectrometry and high-resolution mass spectrometry. The liver, muscle, and viscera of four fish specimens of three different species (one *Lutjanus bohar*, two *Variola louti* and one *Carcharinus leucas*) caught in the waters of La Réunion were analysed. One of the *V. louti* specimens was obtained after a CP incident. Their toxicity values ranged between 0.01 and 2.69 μg CTX1B equiv.·kg$^{-1}$ in flesh, 1.60 and 6.50 μg CTX1B equiv.·kg$^{-1}$ in liver and 0.07 and 4.77 μg CTX1B equiv.·kg$^{-1}$ in viscera. In general, liver and viscera showed higher concentrations of CTX-like compounds than flesh. Several potential CTX congeners were identified. LC-MS/MS confirmed the presence of CTX1B, 52-*epi*-54-deoxyCTX1B and 54-deoxyCTX1B in the flesh of the *V. louti* specimen involved in the CP incident. CTX1B was identified in the three tissues of the other *V. louti* individual and in the *L. bohar*. The *C. leucas* samples revealed the presence of a complex CTXs profile showing congeners of the CTX3C-group. The strategy used within this research work could be a valuable tool for future food safety monitoring in the Indian Ocean area.

## 1. Introduction

Ciguatera poisoning (CP) is a frequent seafood illness caused by the consumption of marine fish contaminated with ciguatoxins (CTXs) (Chinain et al., 2020; FAO, 2020) and is prevalent in circumtropical regions of the world, including areas of the Pacific Ocean, Indian Ocean, Caribbean Sea, and Gulf of Mexico (Bagnis et al., 1980; Tester et al., 2010). CTXs are marine biotoxins produced by dinoflagellates of the genera *Gambierdiscus* (Chinain et al., 2020; Holmes et al., 1991; Litaker et al., 2017; Roeder et al., 2010) and *Fukuyoa* (Laza-Martinez et al., 2016; Litaker et al., 2017, Murray et al., 2024). These toxins are lipophilic polyether molecules that have been associated with gastrointestinal, neurological, and cardiovascular symptoms after the consumption mainly of fish (Bagnis et al., 1979; Boisier et al., 1995; Habermehl et al, 1994; Pearn, 2001; Rabenjarison et al., 2016). Transfer, bioaccumulation and biomagnification of CTXs occurs in the food webs as small herbivorous and omnivorous reef fish graze on the toxic microalgae, which are then predated by larger omnivorous and carnivorous fish (Mak et al., 2013). During transfer of toxins through the food webs, bio-transformation processes result in a variety of new toxic compounds (Ikehara et al., 2017).

CTXs represent a structurally diverse toxin class with more than

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thirty different congeners (FAO and WHO, 2020) that are categorized into four different groups based on their chemical structures and the place of occurrence: the CTXs from the Pacific Ocean namely P-CTXs (Satake et al., 1998; Yasumoto et al., 2000) are actually sub-divided into two separate groups (CTX3C-group and CTX4A-group), based on the algal precursors leading to the congeners of the respective groups (Murata et al., 1990; Satake et al., 1993; Satake et al., 1998; Yasumoto et al., 2000). The Caribbean CTXs represent the third group (C-CTX1-group) (Vernoux & Lewis, 1997) and the Indian CTXs represent the fourth group (I-CTX1-group) (Hamilton et al., 2002a; 2002b).

Five distinct structures have been identified within the C-CTX1-group. Nevertheless, the existence of additional congeners is suggested by bioassay-guided fractionation and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis (Estevez et al., 2019; Kryuchkov et al., 2020; Lewis et al., 1998; Mudge et al., 2023; Mudge et al., 2024; Pottier et al., 2002; Spielmeyer et al., 2022; Estevez et al., 2024). Indian CTXs are the least well-characterized group, and their complete structural elucidation remains unresolved to date. In a shark tissue sample from Madagascar, four potential and two putative I-CTX congeners were identified using liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) (Diogène et al., 2017). The structure of I-CTXs is believed to resemble that of Caribbean CTXs, as both congeners share the same molecular formula and exhibit similar retention times (Diogène et al., 2017; Hamilton et al., 2002a; Spielmeyer et al., 2022).

Due to the lack of standards and reference materials, the lipophilic characteristics and low levels of CTXs in fish, the large difference in polarities, in addition to minor modifications added by fish metabolism and differences in the type of dinoflagellates produced by these two groups (CTX3C- and CTX4A-group), great challenges arise during the analyses with LC-MS/MS, especially when the CTX3C- and CTX4A-groups coexist (Chinain et al., 2020; FAO, 2020).

There is a clear global need for the improvement of analytical tools to help with the identification of toxins produced by *Gambierdiscus* and *Fukuyoa* as well as those present in toxic fish, and with their distribution in tissues, and their bio-transformation processes through the food web (FAO, 2020, Murray et al., 2024; Oshiro et al., 2021). Additionally, toxicity of CTXs in fish remains poorly understood (Loeffler et al., 2023).

The US Food and Drug Administration (FDA) has set guidance values of 0.01 μg $kg^{-1}$ for CTX1B equivalents and 0.1 μg $kg^{-1}$ for C-CTX1 equivalents in fish samples (Dickey & Plakas, 2010; USFDA, 2011). In addition, the CODEX Committee on Contaminants in Foods (CCCF) is considering the establishment of maximum limits for CTX1B and C-CTX1, and the development of risk management guidelines. To help promote research activities on CP, in 2015 UNESCO formulated an explicit global strategy through its Intergovernmental Panel on Harmful Algal (https://oceanexpert.org/doclist/158). One of the three elements of the strategy involves improved analytical methods for toxin detection in microalgal cells and fish (Murray et al., 2018).

Traditionally, the analysis of CTXs relied on a mouse bioassay (MBA) that was first detailed in the late 1960s (Scheuer et al., 1967). To detect the toxins responsible for CP, methods like cell-based assays (CBAs) (Abraham et al., 2012; Dechraoui et al., 2005; Lewis et al., 2016; Manger et al., 1993; Pisapia et al., 2017; Tudó et al., 2022), receptor binding assays (RBAs) (Darius et al., 2007; Hardison et al., 2016) as well as immunoassays and immunosensors (Gaiani et al., 2020; Leonardo et al., 2020; Tsumuraya et al., 2018; Tudó et al., 2022) were used. However, to discern the chemical nature of the toxins responsible for CP, it has been necessary to develop more specific and selective methods of analysis for CTXs, such as LC-MS/MS (Estevez et al., 2019; Estevez et al., 2023; Lewis et al., 2009; Oshiro et al., 2010; Spielmeyer et al., 2022; Wu et al., 2011; Yogi et al., 2011). LC-MS/MS requires efficient and selective sample processing techniques, to optimise extraction and concentration of toxins, and to remove matrix-derived compounds that negatively impact sample analysis.

The worldwide incidence of CP is estimated at between 10,000 and 500,000 cases per year (Ragelis, 1984, Friedman et al., 2017) although these figures would require update and further re-evaluation. In the Indian Ocean, CP was first described in Mauritius (Halstead & Cox, 1973). Except for some historical cases, very little information is available on both epidemiological incidence in the islands of the area (Madagascar, Comoros, the Seychelles, Mauritius, and Rodrigues) and the ecotoxicological phenomena leading to toxic fish. In 1993, a first possible event of CP was described after the consumption of a shark in Manakara (south-east coast of Madagascar) and was noted for its unprecedented severity. In this CP event, several hundred people (between 200 and 500 depending on the different authors) were poisoned due to the consumption of a shark, either a bull shark *(Carcharhinus leucas*) or a pigeye shark (*Carcharhinus amboinensis*), two species that are difficult to be distinguished (Boisier et al., 1995; Habermehl, et al., 1994); Quod et al., 1994). Another case of poisoning was recorded in Madagascar in 2013 after the consumption of a bull shark (*C. leucas*) which caused the poisoning 97 people and death of 11 people and I-CTXs were confirmed in the stomach of that specimen (ANSES, 2015; Diogene et al., 2017; Rabenjarison et al., 2016).

Groupers are a valuable fishery resource of reef ecosystems and are among the species most vulnerable to fishing pressure, as they are strongly targeted because of their high commercial value (Heemstra & Randall, 1993) and also due to their life history, as they generally tend to form spawning aggregations which are easily targeted by fishers (Stump et al., 2017). Despite their economic importance, few grouper fisheries are regularly monitored and many are reported to be undergoing declines (Frisch et al., 2016; Sadovy de Mitcheson et al., 2013). The sub-family Epinephelinae (Bleeker, 1874 included in the family Serranidae; Swainson, 1839) comprises 127 valid species (Eschmeyer & Fricke, 2022), which are mostly distributed in tropical and subtropical seas (Mahé et al., 2022). Amongst all species of this Epinephelinae sub-family, the five main species in the commercial catches around La Réunion, targeted by artisanal fisheries, are blacktip grouper (*Epinephelus fasciatus*), oblique-banded grouper (*Epinephelus radiatus*), golden hind *(Cephalopholis aurantia*), white-edged lyretail (*Variola albimarginata*), and yellow-edged lyretail (*Variola louti*,) (Biais and Taquet, 1992; Le Manach et al., 2015; Roos et al., 1998). In La Réunion and Mauritius, regulations have long existed to prohibit the sale or import of species considered toxic and a ban has been imposed on the marketing of some toxic species. However, CP outbreaks still occur today in these countries (Quod and Turquet, 1994). Thirty-four species of fish have been identified during the CP episodes in the archipelago of Mascarenes, with large predators such as groupers (Mahé et al., 2022) being the main culprits of this incidence: Serranidae: 53 % (*V. louti, Plectropomus* spp., *Epinephelus* spp.); Lutjanidae: 6 % (*Lutjanus bohar, Lutjanus sebae*); Carangidae: 10 % (*Caranx melampygus, Carangoides fulvoguttatus*); and Lethrinidae: 15 % (*Monotaxis grandoeulis, Lethrinus* spp.) (Quod & Turquet, 1994).

Toxicities and toxin profiles of fish from the Indian Ocean are poorly documented. In this work, a multidisciplinary study on the toxicity by neuro-2a CBA, the presence of CTX-like compounds by a magnetic bead-based immunoassay, and the toxin profile by LC-MS/MS and LC-HRMS were implemented for three fish species (*V. louti, L. bohar* and *C. leucas*) from La Réunion. *V. louti* and *L. bohar* are the most frequently fish species involved in CP in this region and *C. leucas* was involved in fatal poisoning cases.

## 2. Material and methods

### 2.1. Standards and reagents

CTX1B, CTX4A, CTX3C, CTX3B, 2,3-dihydroxyCTX3C, and M-secoCTX3C were kindly supplied by Mireille Chinain from Institute Louis Malardé (Tahiti); 51-hydroxyCTX3C was kindly supplied by Prof. Takeshi Yasumoto (Japan Food Research Laboratories); 52-*epi*-54-deoxyCTX1B (P-CTX2) and 54-deoxyCTX1B (P-CTX3) were purchased

from Professor R. J. Lewis (The Queensland University, Brisbane, Australia); and gambierone (Lot 20-001), and 44-methylgambierone (Lot 20-001) were purchased from CIFGA (Lugo, Spain). Internal reference materials were used for identification of some analogues: C-CTX1 was detected in a *Seriola* sp. from the Canary Island from the Eurocigua project and C-CTX1/2 and C-CTX3/4 in a barracuda extract as a gift from Alison Robertson (University of South Alabama, USA); I-CTX1/2, and I-CTX3/4 were detected from the Madagascar shark after the analyses by LC-HRMS in previous studies with CITEB (Diogene et al, 2017). All solutions were stored in glass vials at -20 °C. Mixed standards solutions were prepared in methanol and stored in glass vials at -20 °C. CTX1B standard was used for Neuro-2a CBA, immunoassay, and LC-MS/MS analysis quantification. 51-hydroxyCTX3C standard was also used for immunoassay quantification. All the other standards were used only for LC-MS/MS analysis identification and method optimization.

Neuroblastoma murine (Neuro-2a) cells (cell line CCL-131) were purchased from ATCC LGC standards (Manassas, VA, USA). Poly-L-lysine, foetal bovine serum (FBS), L-glutamine solution, ouabain (O), veratridine (V), phosphate buffered saline (PBS), penicillin, streptomycin, RPMI-1640 medium, sodium pyruvate, thiazolyl blue tetrazolium bromide (MTT) and acetone, water, methanol, hexane, diethyl ether, and ethyl acetate used for sample preparation were HPLC grade (Merck KgaA, Darmstadt, Germany). Dimethyl sulfoxide (DMSO) was purchased from Honeywell (Badalona, Spain).

Dynabeads M-270 Carboxylic Acid, containing $2 \times 10^9$ beads/mL, were obtained from Invitrogen (Life Technologies, S.A., Alcobendas, Spain). Potassium phosphate monobasic, potassium phosphate dibasic, Tween-20, bovine serum albumin (BSA), 4-morpholineethanesulfonic acid (MES) hydrate, N-(3-(dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), and 3,3′,5,5′-tetramethylbenzidine (TMB) liquid substrate were purchased from Sigma-Aldrich (Tres Cantos, Spain). PolyHRP-streptavidin, at 0.5 mg/mL, was obtained from Thermo Fisher (Barcelona, Spain).

Water and acetonitrile were LC-MS/MS grade (Merck KgaA), and formic acid and ammonium formate used to prepare mobile phases were LC-MS/MS grade (Sigma Aldrich, Saint Quentin Fallavier, France).

### 2.2. Fish samples

Sampling was performed at various locations of the Indian Ocean close to La Réunion. The method of capture in Mauritius and Réunion for these species was manual deep vertical longline fishing. Dissection was performed at CITEB (La Réunion), and fish were maintained at -20 °C until transferred in dry ice to the Institute of Agrifood Research and Technology (IRTA) laboratories in La Ràpita, Tarragona (Spain). The fish involved in the CP poisoning was bought in Mauritius Island and consumed in La Réunion. The remains of this toxic meal were also transferred to IRTA.

These samples were received frozen and were stored at -20 °C until extraction. Fish samples included three different species of the genera *Lutjanus, Variola* and *Carcharhinus*. Liver, flesh, and viscera (intestinal track) of three fish samples (one *L. bohar*, one *V. louti*, and one *C. leucas*) were collected. Only flesh was used for *V. louti* (15/41). Fish codes and tissues are summarized in Table 1.

### 2.3. Extraction of fish samples

Fish tissues were processed using an extraction protocol based on the publication of Tudó et al. (2022). Briefly, the four fish were dissected and flesh, viscera, and liver were obtained (10 ± 0.1 g), each tissue was weighted in 50 mL Falcon tubes. Fish tissues were kept at −20 °C until toxin extraction. To perform the extraction, the sample was heated at 70 °C for 10 min in a water bath. Then, an acetone homogenization (2 mL $g^{-1}$ wet weight of tissue) was carried out with an Ultra-Turrax blender (IKA, T25 Basic, Staufen, Germany) for 3 min. The homogenate was centrifuged at 3000 g for 15 min at room temperature (25 °C), and the supernatant was recovered and passed through a 0.22 μm PTFE filter (Whatman, Sigma-Aldrich). The acetone homogenization was repeated twice. The supernatants were pooled and evaporated in a Syncore® Polyvap evaporator (Büchi R-200, Flawil, Switzerland) at 40 °C.

After drying the acetonic phase, the aqueous residue was adjusted with Milli-Q water to 4 mL and diethyl ether was added, mixed by vortex, to initiate a water: diethyl ether partition (1:4, *v:v*) and kept at room temperature for 24 h. Afterwards, the diethyl ether phase was recovered and dried under $N_2$ gas (Turbovap, Zymark corp, Hopkinton, MA, USA) at 40 °C. The dried extract was dissolved in methanol: water (8:2, *v: v*) and partitioned three times with *n*-hexane (1:2, *v: v*). The hexane layers were discarded, and the methanolic phase was dried with the evaporator at 60 °C. Finally, the resulting residues were re-dissolved in 4 mL of HPLC-grade methanol and preserved at −20 °C (protocol based on Yogi et al., 2011 with slight modifications). This crude extract was used by Neuro-2a CBA, magnetic bead-based immunoassay, LC-MS/MS and LC-HRMS analysis.

### 2.4. CTX-like toxicity evaluation by Neuro-2a CBA

The cytotoxicity tests of the extracts were carried out using CTX1B standard as a reference. The evaluation of CTX-like toxicity was performed using the Neuro-2a CBA following the procedure previously described (Caillaud et al., 2012; Soliño et al., 2015). Neuro-2a cells were maintained in RPMI-1640 medium supplemented with 10 % *v/v* FBS (Sigma-Aldrich, St. Louis, MO, USA), 1 % *v/v* of 100 mM sodium pyruvate solution (Sigma-Aldrich) and 1 % *v/v* of penicillin-streptomycin (Sigma-Aldrich) at 37 °C in a 5 % $CO_2$ humidified atmosphere.

Cells were seeded in treated 96-well plates (flat bottom) at 34,000 cells/well in 200 μL of RPMI-1640 medium containing 5 % *v/v* FBS and

**Table 1**
List of individuals captured in the coastal waters of La Réunion. Species were determined by morphological identification. FB, feeding behaviour (C: carnivorous); G: gender (F: female); ND: non-determined.

| Code | Common name | Species | Location | Date of capture | Weight (kg) | Length (mm) | FB | G | GPS |
|---|---|---|---|---|---|---|---|---|---|
| 02/DIV/36 | *Yellow-edged lyretail* (EN) *Croissant queue jaune* (FR, RE) | *V. louti* | La Réunion (Mont La Pérouse) | 14/01/2003 | 7,5 | ND | C | ND | -19.72448 / 54.14543 |
| 15/41* | *Yellow-edged lyretail* (EN) *Croissant queue jaune* (FR, RE) | *V. louti* | Mauritius | 25/03/2015 (consumption date) | 5,7 | ND | C | ND | -20.237 / 57.630 |
| 02/DIV/38 | *Two-spot red snapper* (EN) *Vivaneau chien rouge* (FR) *Vara vara* (FR, RE) | *L. bohar* | La Réunion (Mont La Pérouse) | 21/01/2003 | 7,0 | ND | C | ND | -19.72448 / 54.14543 |
| 20/10 | *Bull shark* (EN) *Requin bouledougue* (FR, RE) | *C. leucas* | La Réunion | 08/01/2020 | 150 | 2670 | C | F | -21.05345 /55.21401 |

*This fish was involved in a CP case in 2015.

incubated at 37 °C in a 5 % $CO_2$ humid atmosphere for 24 h. After 24 h, ouabain and veratridine (O/V) were added to final concentrations of 120 μM and 12 μM respectively, and 10 μL of each sample (1/2 serial dilutions of extract or standard) were added to each well. After 24 h, cell viability was measured using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] colorimetric assay (Manger et al., 1993). Absorbance was measured at 570 nm using an automated plate spectrophotometer (BioTek Synergy LX Multimode Reader, USA). Samples were analysed in triplicate. Dose-response curves were fitted to a sigmoidal logistic four-parameter equation using SigmaPlot software 12.0 (Systat Software Inc., USA).

The limit of detection/quantification (LOD/LOQ) for CTX1B standard was defined as the amount of CTX1B that causes 20 % of inhibition of cell viability ($IC_{20}$) in O/V + and that can be expressed according to the maximum concentration of tested tissue that does not affect the viability of Neuro-2a cells under O/V – conditions (Caillaud et al., 2010). When implementing the exposure of cells to fish extracts, the LOD/LOQ for a specific fish (as reported in Table 2) was determined according to the LOD/LOQ of the CBA and the maximum concentration of fish extract where no matrix effect was observed. Matrix effect was considered when toxicity was recorded following extract exposure in the absence of O and V treatment (O/V-). CTX-like toxicity, estimated with calibration curves and concentration response curves, was quantified by inferring the concentration of CTX1B causing a cell viability inhibition of 50 % ($IC_{50}$) and the concentration of extract resulting in a 50 % cell viability inhibition.

### 2.5. CTX-like compounds evaluation by magnetic bead-based immunoassay for CTXs

The magnetic bead-based immunoassay previously described (Leonardo et al., 2020) was applied to the fish samples to assess the presence of CTX-like compounds, following the strategy to discriminate between the CTX1B and CTX3C series (Gaiani et al., 2020).

Initially, the 3G8 and 10C9 monoclonal antibodies (mAbs) were immobilized on magnetic beads (MBs), obtaining two different conjugates: 3G8-MB (Tsumuraya et al., 2012) and 10C9-MB (Oguri et al., 2003). To produce 1 mL of conjugate, 100 μL of MBs was transferred to a tube and washed twice with 25 mM MES, pH 5.0 (MES). For the washing steps, the tube was placed on a magnetic separation stand to remove supernatants. The MBs were then incubated with 0.5 mL of EDC at 25 mg/mL and 0.5 mL of NHS at 25 mg/mL in MES for 30 min. After washing twice, 1 mL of 3G8 and 10C9 mAb at 1/50 dilution in MES was added and incubated for 1 h. Finally, the mAb-MB conjugates were washed three times with 0.1 M PBS, 0.05 % Tween-20 (*v/v*), pH 7.2 (PBST) and resuspended in 1 mL of the same buffer.

For the immunoassay: 35 μL of 3G8-MB or 10C9-MB conjugates were put on the magnetic separation stand and the supernatant was removed. Then, 35 μL of CTX1B standard solution or fish extract (previously evaporated and resuspended in PBST) was added and incubated for 30 min; after three washing steps with PBST, a blocking step was performed with PBST with 2 % BSA (*w/v*) (PBST-BSA) for 30 min; the conjugates were washed three times with PBST and 35 μL of biotin-8H4 mAb (Tsumuraya et al., 2006) in PBST-BSA at 1:2000 dilution was added and incubated for 30 min; three washing steps were performed with PBST, and 35 μL of polyHRP-streptavidin in PBST-BSA at 1:1000 dilution was added and incubated for 30 min; finally, three washing steps were performed in PBST and the content of each tube was resuspended in 35 μL of the same buffer. For colorimetric measurement: 10 μL of immunocomplexes was transferred to a new tube, and the supernatant was removed; 125 μL of TMB was added, followed by a 10 min incubation; tubes were placed on the magnetic separation stand, and 100 μL of solution was taken for absorbance reading at 620 nm. All incubations were performed at room temperature and with slow tilt rotation. Measurements were performed in triplicate. Dose-response curves were blank-subtracted and fitted to a sigmoidal logistic four-parameter equation using SigmaPlot software 12.0 (Systat Software Inc., USA).

The LOD and LOQ were calculated using 3 and 10 times the standard deviation of the blank value (no CTX) and were determined to be 0.001 μg CTX1B or 51-hydroxyCTX3C equiv.·$kg^{-1}$ and 0.004 μg CTX1B or 51-hydroxyCTX3C equiv.·$kg^{-1}$, respectively. The investigation of matrix effects was conducted through the analysis of serial dilutions of each fish extract. The stabilisation of the CTX contents in two successive fish extract dilutions indicates absence of matrix effects. The CTX contents in each dilution were quantified by comparing the absorbance values obtained with those from the analysis of different doses of the CTX1B standard and 51-hydroxyCTX3C standard, respectively.

### 2.6. Clean-up of fish samples

After extraction of fish tissue, a clean-up by solid phase extraction (SPE) was carried out to minimise all possible interferences and reduce the matrix effects when analysing crude extracts, which could interfere with LC-MS/MS and LC-HRMS analysis (Estevez et al., 2019). Clean-up was carried under three conditions: (A) Normal phase Florisil SPE (J. T. Baker, 500 mg, Center Valley, PA, USA) was used to remove polar interfering compound, 2 mL of sample extract in ethyl acetate (AcOEt) was loaded in a cartridge previously conditioned with 3 mL of AcOEt, then washed with 3 mL of AcOEt and eluted with 10 mL AcOEt:MeOH (9:1); Florisil purified extract was evaporated and reconstituted in 250 μL of MeOH; (B) Reversed phase C18 SPE (J. T. Baker, 500 mg, Center Valley, PA, USA) was used to remove non-polar and semipolar interfering matrix constituents; C18 cartridge was conditioned with 3 mL MeOH:$H_2O$ (60:40) to load 2 mL of sample extract in MeOH:$H_2O$ (60:40); then, the cartridge was washed with 3 mL MeOH:$H_2O$ (60:40) and eluted with 10 mL MeOH:$H_2O$ (90:10) and 10 mL MeOH (100 %); C18 purified extract was evaporated and reconstituted in 250 μL of MeOH; and (C) Combination of normal phase Florisil SPE and reversed phase C18 SPE was used to remove polar and non-polar compounds and was carried out in the same conditions described in A and B, with the difference that condition A was continued with previously evaporating the purified extract of Florisil SPE and dissolved it in MeOH:$H_2O$ (60:40). All extracts were filtered (Syringe Driver filter Unit, Millex®-CV 0.22 um, 13 mm, Millipore, Billerica, MA, USA) prior to the analysis by both LC-MS/MS and LC-HRMS.

### 2.7. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analysis

An Acquity UPLC I-Class coupled to a Xevo TQ-XS triple quadrupole mass spectrometer (Waters, Milford, MA, USA) with flow through needle sample manager was used for the analysis by LC-MS/MS. The chromatographic separation was performed on a reversed-phase BEH C18 (50 mm × 2.1 mm, 1.7 μm) (Waters) at a flow rate of 400 μL $min^{-1}$. Mobile phase A was water, and B was acetonitrile/water (95:5), both containing 2 mM ammonium formate and 0.1 % formic acid. The gradient elution program for the analysis was: 5 % B 0 min, 50 % – 50 % B 1 min, 100 % B 5 min, hold 100 % B until 7 min and return to initial conditions for re-equilibration (4 min 5 % B). A 2-μL injection volume was used. The total duration of the method was 11 min.

Five LC-MS/MS analysis methods were used for CTX identification and confirmatory purposes (Tables S1 and S5) in order to cover the main congeners of CTX4A-group and CTX3C-group, C-CTX1-group and I-CTX1-group. The LC-MS/MS method described in Table S1 was used for quantification purposes. For the mass spectrometer, the MS method operated in positive ionization mode using electrospray ionization source (ESI) monitoring the MS/MS transitions, collision energy (CE) as well as cone voltage for each precursor/product ion, which are summarized in Table S1-S5.

The MS/MS transitions for the various CTXs were determined from the injection experiment of toxin standards at different collision energy and cone voltage. Both were optimized for each compound monitored.

CTX4B was incorporated based on results in the literature and the acquisition of CTX4A. A dwell time of 20 ms was used for the CTXs transitions monitored. Capillary voltage 3.0 kV, cone voltage 50 V, source temperature 150 °C, nitrogen gas desolvation flow rate was 1000 L $h^{-1}$ at 600 °C, cone gas 150 L $h^{-1}$, and the collision cell was operated with 0.15 mL $min^{-1}$ argon.

Data acquisition and processing was performed with TargetLynx software (Waters, Milford, US). Identification and confirmation were supported by toxin retention time and MRM ion ratios in order to avoid false positives.

#### 2.7.1. SPE recovery rates and matrix effect

SPE recovery rates were determined using negative *L. bohar* (flesh) and *C. leucas* (flesh, liver, and viscera) (previously analysed by LC-MS/MS) in duplicate. Each sample was spiked at with 1.25 ng $mL^{-1}$ of CTX1B, corresponding to a content of 0.5 μg $kg^{-1}$ for fish sample. The spiked extracts were incubated at room temperature for 10 min to allow interaction of the standards with the matrix, followed by the three clean-up protocols described in section 2.6. The samples were analysed in duplicate. SPE recovery rates for individual injections were calculated according to Eq. (1):

$$Recovery\ (REC)(\%) = \frac{C(measured)}{C(theorical)} \times 100 \quad (1)$$

with C (*theorical*) corresponding to the theoretical concentration in the final extract. For each matrix, the mean of the individual recovery rates (n = 2) was used.

In order to evaluate the matrix effect of the *L. bohar* and *C. leucas* matrices, the same tissues used in the recovery evaluation were tested. Blank extracts were cleaned up using the three protocols described in section 2.6. Each SPE eluate was spiked with 1.25 ng $mL^{-1}$ of CTX1B, corresponding to a content of 0.5 μg $kg^{-1}$ for fish sample. Samples were analysed in duplicate. The matrix effect of individual injections was calculated according to Eq. (2):

$$Matrix\ effect\ (ME)(\%) = \frac{C(measured)}{C(theorical)} \times 100 \quad (2)$$

with C (*theorical*) corresponding to the theoretical concentration in the final extract (10 ng·$mL^{-1}$). The mean of matrix effect (n = 2) was used.

#### 2.7.2. Limit of detection and limit of quantification

LODs, LOQs and linearity were determined in solvent and extracts of *L. bohar* (flesh). Solvent extracts and *L. bohar* extracts were prepared in methanol. LODs and LOQs were determined by the signal-to-noise ratio (S/N) and triplicate injection. LOD and LOQ were defined as S/N ≥ 3 and S/N ≥ 10, respectively (Wenzl et al., 2016).

For LOD/LOQ determination in matrix samples, the *L. bohar* and *C. leucas* extracts prepared for recovery determination (Section 2.7.1) were diluted with a *L. bohar* and *C. leucas* blank matrix at 1:2, 1:5, 1:10 and 1:20 (*v/v*) ratios. Samples were analysed using the screening method with two transitions.

#### 2.7.3. Precision and repeatability

For determining the repeatability, a naturally contaminated fish flesh sample of *L. bohar* (02/DIV/38) was utilized. Six flesh samples from the same specimen were extracted, prepared, and analysed in duplicate. Repeatability was calculated as the relative standard deviation ( % RSD), based on peak areas of CTX1B.

### 2.8. Liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) analysis

An Orbitrap-Exactive HCD (Thermo Fisher Scientific, Bremen, Germany) mass spectrometer equipped with heated electrospray source (H-ESI II), a Surveyor MS Plus pump and an Accela Open AS auto-sampler kept isothermal at 15°C (Thermo Fisher Scientific, San Jose, California) was used for the analysis by LC-HRMS.

The chromatographic separation was performed on a reversed-phase Hypersil Gold C18 (100 mm × 2.1 mm, 1.9 μm) (Thermo Fisher, Scientific, Bremen, Germany) at a flow rate of 250 μL $min^{-1}$. Mobile phase A was water, and B was acetonitrile/water (95:5), both containing 2 mM ammonium formate and 0.1 % formic acid. The gradient elution program for the analysis was: 30 % B1 min, 30 %–40 % B 2 min, 40 %–50 % B 1 min, 50 %-90 % B 5 min, 90 % B 3 min and return to initial conditions to re-equilibrate (2 min) and maintain these initial conditions for 11 min. A 5 μL injection volume was used. The total duration of the method was 25 min.

An external calibration was carried out from 1 to 50 ng $mL^{-1}$ (corresponding 0.05 to 2.5 μg equiv CTX1B · $kg^{-1}$ in tissue) using CTX1B and showed good linearity ($R^2$ = 0.9997), with the LOD being 0.4 ng $mL^{-1}$(0.02 μg equiv CTX1B· $kg^{-1}$ in tissue). In order to calculate concentrations of CTX analogues, it was assumed that related analogues would give a similar response to that obtained by the CTX1B standard.

The analyses were carried out in positive electrospray ionization (ESI+) mode, and the instrument was calibrated daily. The parameters in positive mode (ESI+) were a spray voltage of 4.0 kV, capillary temperature of 275 °C, heater temperature of 300 °C, sheath gas flow rate of 35 psi and auxiliary gas flow rate of 10 (arbitrary units). In addition, capillary voltage of 47.5 V, tube lens voltage of 186 V and skimmer voltage of 18 V were used. Nitrogen (purity > 99.999 %) was employed as sheath gas, auxiliary gas, and collision gas. The mass range was 400–1,500 *m/z* in full scan acquisition mode. The resolution was 50.000 (200 *m/z*, FWHM) at a scan rate 2 Hz, the automatic gain control (AGC) was set as "balanced" ($1 \times 10^6$) with a maximum injection time of 250 ms. The data was processed with Xcalibur 2.2 SP1 software (Thermo Fisher Scientific, Bremen, Germany).

The peaks were extracted from the chromatogram using the sum of exact mass of $[M+H]^+$, $[M+NH_4]^+$ and $[M+Na]^+$ diagnostic and confirming ions with the ±10 ppm of mass accuracy extraction window. The results were expressed as the sum of the three signals to improve sensitivity and harmonize the results regardless of the different relative intensity obtained for the three ions for each CTXs and for several matrices. In addition to retention time, HRMS and mass accuracy parameters for accurate mass measurements (AMM), in the present study, to be confident in the identification and the proposed elemental formula, the following restrictive criteria were applied: elements considered were restricted in accordance with CTXs molecular formula and adduct signals [C: 55 to 70, H: 64 to 110, O: 11 to 25, N: 0 to 1, and cations (Na): 0 to 1]; the isotopic pattern was matched to theoretical in silico approach and the charge, the ring double bond equivalents (RDBEs) and nitrogen rule were taken into account. Additionally, the mono-isotopic pattern (M+1 ion) of these signals was used to assist in the further confirmation of the toxin identity as a supplementary identification point. Therefore, in total three signals, a diagnostic and two confirming ions, were used for toxin identification. Mass accuracy criteria was < 8.5 ppm. The relative ion intensities between the main signal ($[M+H]^+$, $[M+NH_4]^+$ or $[M+Na]^+$) and their M+1 ions were calculated and matched taking into account a tolerance of 40 % according to the EU Commission SANTE/2021/808 guidance document (Document N° SANTE/2021/808, 2021). The characteristic isotopic pattern, M+1/M ion ratio, is a robust criterion that for all described CTXs is a theoretical value of 0.6–0.7.

The combination of high resolution, mass accuracy, and restrictive criteria was crucial for the identification of both targeted and unknown compounds.

## 3. Results

### 3.1. CTX-like toxicity evaluation by Neuro-2a CBA

Exposure of the Neuro-2a CBA cell line to a CTX1B standard when

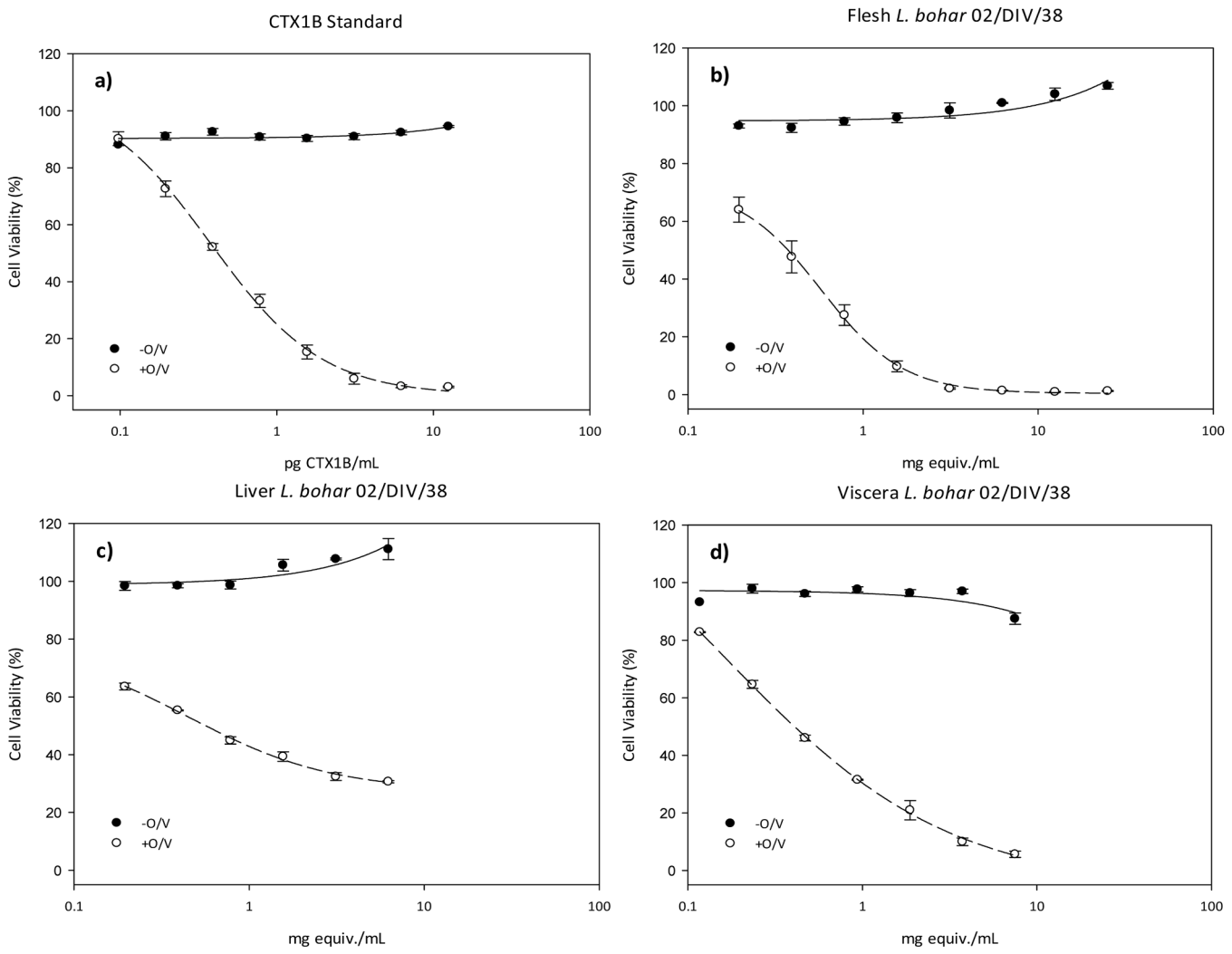


**Fig. 1.** Concentration-response curves of Neuro-2a CBA to CTX1B or liver fish extract, in the absence (O/V -) and presence (O/V+) of O and V: (a) Concentration-response curve of Neuro-2a cells after exposure to CTX1B standard; (b) Dose-response curve of Neuro-2a cells after the exposure to (b) flesh fish extract, (c) liver fish extract and (d) viscera fish extract (*L. bohar* (02/DIV/38)). Data represent the average and the standard deviation (SD) for three replicates on the same day of experimentation.

adding O and V (O/V+) resulted in a typical dose-response curve of CTX-like toxicity, with an average across the experiments LOQ of 0.21 ± 0.05 pg CTX1B·mL$^{-1}$ and an $IC_{50}$ of 0.91 ± 0.45 pg CTX1B·mL$^{-1}$. The ten fish samples were analysed by Neuro-2a CBA, and all exhibited CTX-like toxicity. Results are presented in Table 2. Fig. 1 shows the results obtained in the analysis of a *L. bohar* (02/DIV/38) flesh, liver and viscera with Neuro-2a CBA, as an example. The liver of *C. leucas* (20/10) was the most toxic sample, see Figure S1. In the case of *V. louti* (02/DIV/36), the viscera exhibited the highest toxicity, followed by the liver and the flesh. In contrast, in *L. bohar* (02/DIV/38), the tissue that exhibited the highest toxicity was the flesh, followed by the liver and the viscera. The CTX-like toxicity observed in *L. bohar* (02/DIV/38) represented the highest value

**Table 2**
CTX-like toxicity evaluation using the Neuro-2a CBA and CTX-like compounds evaluation using a magnetic bead-based immunoassay (muscles, livers, and viscera). Quantifications and LOQs are expressed for CBA as μg CTX1B equiv. kg$^{-1}$, and for immunoassay as μg CTX1B or CTX3C equiv. kg$^{-1}$.

| Code | Species | Tissue | Neuro-2a CBA (μg CTX1B equiv·kg$^{-1}$) | Neuro-2a CBA LOQ (μg CTX1B equiv·kg$^{-1}$) | Immunoassay (μg CTX1B equiv·kg$^{-1}$) | Immunoassay (μg 51-hydroxyCTX3C equiv·kg$^{-1}$) |
|---|---|---|---|---|---|---|
| 02/DIV/36 | *V. louti* | Flesh | 0.68 | 0.36 | 0.31 | – |
| | | Liver | 1.60 | 0.01 | 0.58 | – |
| | | Viscera | 4.77 | 0.04 | 0.16 | – |
| 15/41 | *V. louti* | Flesh | 1.23 | 0.23 | 1.15 | – |
| 02/DIV/38 | *L. bohar* | Flesh | 2.69 | 0.06 | 0.60 | <LOQ |
| | | Liver | 2.31 | 0.04 | 0.78 | – |
| | | Viscera | 1.00 | 0.02 | 0.52 | – |
| 20/10 | *C. leucas* | Flesh | 0.01 | 0.005 | – | – |
| | | Liver | 6.50 | 0.08 | – | 0.04 |
| | | Viscera | 0.07 | 0.003 | – | – |

Note: "-" means not detected (<LOD); "<LOQ" means detected but not possible to quantify; LOD immunoassay: 0.001 μg CTX1B or 51-hydroxyCTX3C equiv.·kg$^{-1}$; LOQ immunoassay: 0.004 μg CTX1B or 51-hydroxyCTX3C equiv.·kg$^{-1}$.

observed in the flesh in this study. Regarding the flesh and viscera in *C. leucas* (20/10), a very low CTX-like toxicity was detected in comparison to the other samples.

### 3.2. CTX-like compounds evaluation by magnetic bead-based immunoassay

A CTX1B dose-response curve was constructed with the immunoassay (Fig. 2). Results for the analysis of all fish extracts are shown in Table 2 and the analyses of the different tissues of *L. bohar* (02/DIV/38) are shown in Fig. 2, as example. The presence of CTX1B-like compounds was confirmed in all the tissues of *V. louti* (02/DIV/36 and 15/41) and *L. bohar* (02/DIV/38). However, no CTX1B-like compounds were detected in any of the tissues of *C. leucas* (20/10). Regarding CTX3C-like compounds, only trace amounts were detected in the flesh of *L. bohar* (02/DIV/38) and in the liver of *C. leucas* (20/10) when analysed at 2500 mg/mL (results not shown in Fig. 2), but no signals were obtained in diluted extracts. According to these results, only in the flesh of *L. bohar* (02/DIV/38) co-occurrence of CTX-like compounds from the CTX1B and CTX3C series was described. In the other fish samples, only CTXs from one CTX series are present. The tissue with the highest CTXs contents determined with the immunoassay was the flesh of *V. louti* (15/41). The liver of *V. louti* (02/DIV/36) and the three tissues of *L. bohar* (02/DIV/38) presented similar CTXs contents. The CTXs contents detected in the flesh and viscera tissues of *V. louti* (02/DIV/36) were lower than those observed for other tissues of this work.

### 3.3. CTXs analysis by LC-MS/MS

The samples were analysed by LC-MS/MS using the Multiple Reaction Monitoring (MRM) acquisition mode, selecting two or three transitions per toxin to allow quantification ($MRM_1$; the most intense transition) and confirmation ($MRM_2$ and $MRM_3$).

Source and interface conditions were optimized using CTX standards and mobile phases in ACN/$H_2O$ gave rise to prominent $[M+H]^+$, $[M+NH_4]^+$, $[M+Na]^+$ and serial dehydrated ions. The identification of CTXs was made using reference standards (MRI mix solution) by comparing the retention times, MRM ion ratios and signal-to-noise ratios. The MRI mix solution concentration was adjusted to 10 ng $mL^{-1}$ for CTX1B, 52-*epi*-54-deoxyCTX1B, 54-deoxy-CTX1B, CTX4A, gambierone and 44-methyl gambierone; 40 ng $mL^{-1}$ for CTX3C, CTX3B and 80 ng $mL^{-1}$ for 2,3-diOH CTX3C and M-seco CTX3C.

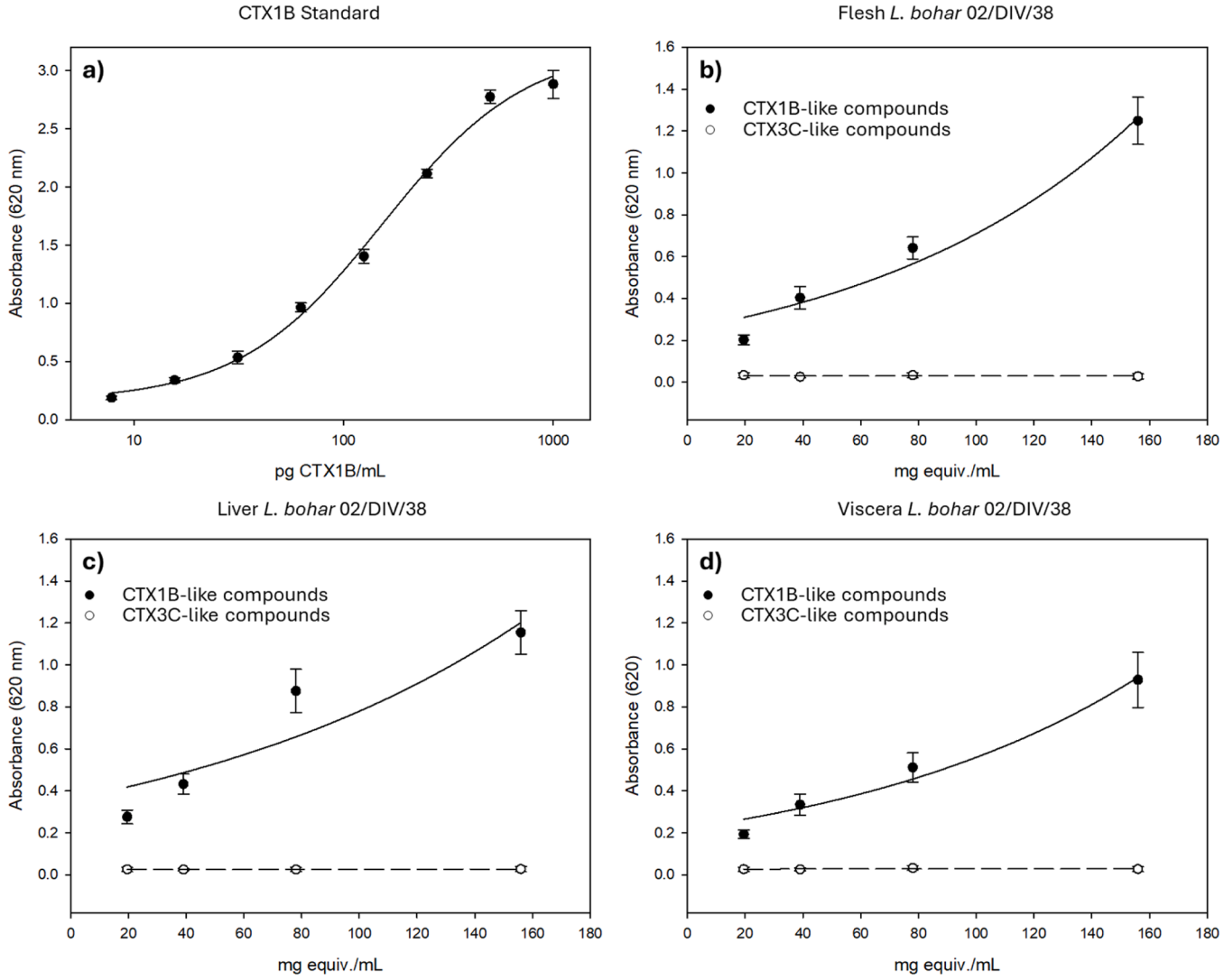


**Fig. 2.** Signals obtained with the immunoassay after exposing the MB conjugates to different concentrations of CTX1B standard (a) and different fish extracts dilutions corresponding to the flesh (b), liver (c) and viscera (d) of *L. bohar* (02/DIV/38). Data represent the average and the standard deviation (SD) for three replicates.

### 3.4. SPE recovery rate and matrix effect evaluation

First, matrix effect (%) and recovery (%) for LC-MS/MS were evaluated spiking CTX1B at 0.5 μg $kg^{-1}$ to *L. bohar* flesh extracts before and after the SPE clean-up protocols (Florisil individually, C18 individually and the combination of Florisil and C18) using an optimized SPE strategy based on (Estevez et al., 2019). A blank extract of *L. bohar* flesh without spiking was necessary to quantify the CTX1B present in the sample and take it into account on the matrix effect (%) and recovery calculations (%). The recovery rates ranged from 81 % to 144 % for CTX1B, while the matrix effect ranged from 61 % to 166 %. All results are summarized in Figure S2.

For Florisil cartridges individually, the matrix effect and recovery exceeded 100 % (166 % and 144 %, respectively). On the contrary, C18 cartridges individually showed a matrix effect and recovery rate of 61 % and 81 %, respectively, indicating that there were losses during the cleaning process. As acceptable recoveries (57.6 %) were described in Estevez et al. (2019) using the combination of Florisil and C18 cartridges, this combination with some little modifications (such as higher elution volumes and additional second elution fraction as described in section 2.6) was also tested in the *L. bohar* flesh, obtaining 83 % of matrix effect and 99 % of recovery rate for CTX1B. Acceptable values were observed; thus, this strategy was selected for further experiments.

Our optimized protocol using the combination of a Florisil and a C18 cartridge was also used for cleaning different matrices of a *C. leucas* sample including flesh, liver and viscera that were subsequently analysed by LC-MS/MS (Figure S3). The recovery rates ranged from 49 % to 89 % and the matrix effect ranged from 81 % to 101 %. Differences among recovery rates are expected, due to the different interfering compounds that could contain these types of matrices. Better recovery rates were observed for the flesh matrix in comparison to viscera and liver. According to the guidance document SANTE/12682/2019 (Document N° SANTE/12682/2019), recovery rates should range between 70 % and 120 %. However, values between 30 % to 140 % can be accepted if the repeatability is below 20 % (see section 2.7.3).

#### 3.4.1. Limit of detection, limit of quantification and linearity

Peak areas were integrated, and sample concentrations calculated from linear calibration curves generated from external calibration solutions of CTX1B between 0.28 – 28 ng $mL^{-1}$, corresponding to 0.014 – 1.4 μg equiv. CTX1B·$kg^{-1}$ in tissue, showed good intra-batch performance and linear adjustment ($R^2$) > 0.9992. To ensure that the slope of the calibration curve showed a linear regression, the correlation coefficient ($R^2$) should be ≥ 0.98.

LOD and LOQ values in solvent were estimated by the calibration method using standards in the concentration range lower and through S/N, with an average value of 0.045 ng $mL^{-1}$ and 0.137 ng $mL^{-1}$, respectively.

LOD and LOQ values in the presence of matrix were determined in diluted extracts obtained from spiked fish samples (see section 2.7.2), with an average LOD of 0.01 μg·equiv.$kg^{-1}$ and an average LOQ of 0.04 μg·equiv.$kg^{-1}$. Thus, the determined limits included losses during the sample clean-up, as well as matrix effects during LC-MS/MS analyses. The values obtained are considered comparable to data published recently (Spielmeyer et al, 2022).

#### 3.4.2. Repeatability

A flesh of *L. bohar* naturally contaminated with CTX1B (n: 6) was extracted according to the extraction protocol (mentioned in Section 2.3), and the extracts were analysed in a duplicate injection. The repeatability (expressed as relative standard deviation) was determined for CTX1B present in the sample and RSD values obtained were below 10 %, reflecting good repeatability of the extraction/detection method, according to SANTE/12682/2019 (Document N° SANTE/12682/2019, 2020).

#### 3.4.3. Analyses of naturally contaminated samples by LC-MS/MS

First, the fish extracts without a SPE clean-up step were analysed by LC-MS/MS and all results are summarized in Table 3. The chromatographic identification showed different analogues of CTXs including CTX1B in flesh of *L. bohar* (02/DIV/38) with the highest concentration, followed by *V. louti* (02/DIV/36), and flesh of *V. louti* (15/41) while in the case of the viscera of *L. bohar* (02/DIV/38) the concentration obtained was lower than the LOQ and the ion ratio difference did not comply with the prerequeset criteria to confirm the presence of CTX1B in this sample, and it was discarded.

In the *V. louti* (15/41), 52-*epi*-54-deoxyCTX1B and 54-deoxyCTX1B were identified in its flesh, but the concentration obtained for both analogues was also below the LOQ, and for *C. leucas (*20/10) no CTXs was observed above the LOD in the three tissues of the fish. Other analogues such as CTX3C in flesh of *V. louti* (15/41) and CTX4A in viscera of *L. bohar* (02/DIV/38) were detected; however, they could not be confirmed because the ion ratio did not correspond to that calculated in the standard, and the peaks were discarded avoiding false positives due to interfering matrix compounds.

After the optimized SPE clean-up, chromatographic identification showed a wider chromatographic profile with different CTX analogues including CTX1B, 52-*epi*-54-deoxyCTX1B, 54-deoxyCTX1B and CTX3C. There was no detection of other CTX4A-group and CTX3C group analogues investigated (Tables S2 and S3) that aligned with the literature and/or reference material. The flesh and liver extracts were also analysed for C-CTXs and I-CTXs toxin analogues (Tables S4 and S5), with no detections that aligned with the literature and/or reference material. Due to the diversity of fish species used in this study and acceptable SPE recoveries and matrix effect results (see Section 3.4), the quantification results reported herein have not been adjusted and are reported as quantified from the analytical system. The flesh of *V. louti* (15/41) showed the highest concentration of CTX1B, followed by flesh and liver of *L. bohar* (02/DIV/38). The third fish that showed CTX1B was *V. louti* (02/DIV/36) with lower concentrations in flesh, liver, and slightly lower in viscera. The highest concentration of 52-*epi*-54-deoxyCTX1B was detected in the flesh of *V. louti* (15/41), followed by the flesh of *V. louti* (02/DIV/36) and the liver and flesh of *L. bohar* (02/DIV/38). The 54-deoxyCTX1B analogue was only detected in the flesh of *V. louti* (15/41), which was the fish with the most complex toxin profile of this study. Regarding the C. *leucas* (20/10), no CTX1B analogues were identified, but a low concentration of CTX3C was detected only in the liver tissue. In general, an increase of the signal is clearly observed and more CTX analogues can be confirmed and quantified when cleaning-up the sample with the optimized SPE method. An example of this improvement is shown in Fig. 3, which summarizes the CTX analogues detected in the *V. louti* (15/41) before and after the optimized SPE protocol.

### 3.5. CTXs analysis by LC-HRMS

The spectra of CTXs were dominated by $[M+H-H_2O]^+$, $[M+H]^+$, $[M+NH_4]^+$ and $[M+Na]^+$, which were used for confirmatory purposes. According to the EU Commission SANTE/808/202 guidance document, which includes requirements for identification of analytes and confirmation of results by LC-HRMS for pesticides residues analysis in food and feed, these four signals were used as diagnostic and confirmatory ions (document used as a reference since there is no similar document for the analysis of toxins). Each signal by LC-HRMS supposes two identification points and the LOD for CTX1B was 0.4 ng · $mL^{-1}$ (0.02 μg equiv CTX1B· $kg^{-1}$ in tissue) as described in section 2.8.

All extracts without SPE clean-up were analysed by LC-HRMS Orbitrap. In general, high matrix effects were also observed showing higher ppm errors and overestimation on the quantifications respect to LC-MS/MS analysis. All results are summarized in Table 3. The analogue CTX1B ($C_{60}H_{86}O_{19}$) was identified in the flesh of *V. louti* (15/41) with the highest concentration followed by the flesh of *L. bohar* (02/DIV/38) and *V. louti* (02/DIV/36). Regarding liver and viscera tissues, CTX1B was

**Table 3**
CTX analogues contents (μg equiv. CTX1B·kg$^{-1}$ tissue) in fish by LC-MS/MS and LC-HRMS. Ion composition, theoretical accurate and measured *m/z*, and mass accuracy of main signals obtained from ESI-Orbitrap spectra.

| Code | Species | Tissue | CTX1B | | 52-*epi*-54-deoxy-CTX1B | | 54-deoxy-CTX1B | | CTX3C | | CTX4A | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LC-MS/MS | | | | | | | | | | | | |
| Retention time (min) | | | 2.28 | | 3.30 | | 3.61 | | 4.95 | | 5.01 | |
| MRM1 | | | 1128.6 > 95 | | 1112.7 > 1077.7 | | 1112.7 > 1077.7 | | 1023.6 > 125.1 | | 1061.6 > 125.1 | |
| MRM2 | | | 1128.6 > 109 | | 1112.7 > 1059.7 | | 1112.7 > 1059.7 | | 1023.6 > 155.1 | | 1078.6 > 1043.6 | |
| | | | Without SPE | With SPE | Without SPE | With SPE | Without SPE | With SPE | Without SPE | With SPE | Without SPE | With SPE |
| 02/DIV/36 | *V. louti* | Flesh | 0.05 | 0.09 | – | 0.06 | – | – | – | – | – | – |
| | | Liver | – | 0.09 | – | – | – | – | – | – | – | – |
| | | Viscera | – | 0.05 | – | – | – | – | – | – | – | – |
| 15/41 | *V. louti* | Flesh | 0.04 | 0.40 | <LOQ | 0.24 | <LOQ | 0.20 | – | – | – | – |
| 02/DIV/38 | *L. bohar* | Flesh | 0.07 | 0.20 | – | 0.02 | – | – | – | – | – | – |
| | | Liver | – | 0.19 | – | – | – | – | – | – | – | – |
| | | Viscera | – | – | – | 0.03 | – | – | – | – | – | – |
| 20/10 | *C. leucas* | Flesh | – | – | – | – | – | – | – | – | – | – |
| | | Liver | – | – | – | – | – | – | – | 0.06 | – | – |
| | | Viscera | – | – | – | – | – | – | – | – | – | – |
| LC-HRMS | | | | | | | | | | | | |
| Ion composition (1st) | | | $[M+NH_4]^+$ | | $[M+NH_4]^+$ | | $[M+NH_4]^+$ | | $[M+Na]^+$ | | $[M+Na]^+$ | |
| Theoretical accurate *m/z* | | | 1128.6102 | | 1112.6152 | | 1112.6152 | | 1045.5495 | | 1083.5652 | |
| Measured *m/z* | | | 1128.6115-1128.6188 | | 1112.6169-1112.6196 | | 1116.6146-1112.6204 | | – | | – | |
| Mass accuracy (ppm) | | | -7.91 – 8.65 | -0.52 - 3.3 | -4.64 - 5.01 | 1.53 - 3.94 | – | -0.5 - 4.62 | – | | – | |
| RDBEs | | | 16.5 | 16.5 | 16.5 | 16.5 | 16.5 | 16.5 | – | | – | |
| Ion ratio tolerance % (M+1 ion ratio) | | | -48 % – 17 % | 2 % – 17 % | 1 % – 35 % | 2 % – 10 % | – | -8 % – 1 % | | | | |
| | | | Without SPE | With SPE | Without SPE | With SPE | Without SPE | With SPE | Without SPE | With SPE | Without SPE | With SPE |
| 02/DIV/36 | *V. louti* | Flesh | 0.42 | 0.19 | 0.54 | 0.22 | – | 0.07 | – | – | – | – |
| | | Liver | – | 0.23 | – | – | – | – | – | – | – | – |
| | | Viscera | – | 0.13 | – | – | – | – | – | – | – | – |
| 15/41 | *V. louti* | Flesh | 1.07 | 0.72 | 0.84 | 1.75 | – | 0.63 | – | – | – | – |
| 02/DIV/38 | *L. bohar* | Flesh | 0.86 | 0.85 | – | – | – | – | – | – | – | – |
| | | Liver | 0.62 | 0.39 | – | – | – | – | – | – | – | – |
| | | Viscera | 0.36 | 0.02 | – | – | | – | – | – | – | – |
| 20/10 | *C. leucas* | Flesh | – | – | – | – | – | – | – | – | – | – |
| | | Liver | – | – | – | | – | | – | | – | – |
| | | Viscera | – | – | – | – | – | – | – | | – | – |

Note: "-" means not detected; quantifications for LC-HRMS $\sum [M+H-H_2O]^+ + [M+H]^+ + [M+NH_4]^+ + [M+Na]^+$

detected in the *L. bohar* (02/DIV/38), and both concentrations were lower than the one detected in the flesh of this specimen. The identification of this CTX analogue was confirmed detecting the main adduct *m/z* 1128.6102 $[M+NH_4]^+$, *m/z* 1133.5656 $[M+Na]^+$, *m/z* 1093.5730 $[M-H_2O+H]^+$, and *m/z* 1111.5836 $[M+H]^+$. A second analogue was detected without SPE clean-up, the analogue 52-*epi*-54-deoxyCTX1B ($C_{60}H_{86}O_{18}$), which was identified in the flesh of both *V. louti* specimens, showing higher concentration in the *V. louti* (15/41) in comparison to the *V. louti* (02/DIV/36). The identification of 52-*epi*-54-deoxyCTX1B was confirmed detecting *m/z* 1112.6152 $[M+NH_4]^+$, *m/z* 1117.5706 $[M+Na]^+$, *m/z* 1077.5781 $[M-H_2O+H]^+$, and *m/z* 1095.5886 $[M+H]^+$. The mass accuracy of the main adduct $[M+NH_4]^+$ matched ppm < 5.0, showing quite good mass accuracy. No CTX analogues were detected in the *C. leucas* tissues.

After the optimized clean-up SPE protocol, the same samples were analysed by LC-HRMS. As it was observed by LC-MS/MS (section 3.4.3), the main analogue detected in the fish samples also was CTX1B. There was no detection of other CTX4A-group, CTX3C-group, C-CTXs or I-CTXs analogues investigated. In general, quantifications were lower after the clean-up, except for liver and viscera of *V. louti* (02/DIV/36) where CTX1B was detected, and in addition the mass accuracy was improved matching ppm < 4.0. In Fig. 4a–b, the chromatogram and mass spectra of CTX1B in *V. louti* (15/41) after the clean-up is shown as an example. In contrast to the previous results without SPE, the *L. bohar* (02/DIV/38) flesh showed the highest concentration followed by *V. louti* (15/41) and *V. louti* (02/DIV/36) flesh. The *L. bohar* (02/DIV/38) showed a highest concentration than the *V. louti* (02/DIV/36). *L. bohar* (02/DIV/38) liver showed also the highest concentration followed by *V. louti* (02/DIV/36), however the opposite trend was observed in the viscera of these two specimens.

A little improvement of the mass accuracy was also observed for 52-*epi*-54-deoxyCTX1B analogue after the clean-up, *V. louti* (15/41) flesh showed higher concentration followed by *V. louti* (02/DIV/36). This analogue was not detected in the other two tissues. In the case of 54-deoxyCTX1B, this analogue was only detected following SPE clean-up, in both cases concentration was lower than 52-*epi*-54-deoxyCTX1B following the same trend in the *V. louti* specimens. The chromatogram and mass spectra of 52-*epi*-54-deoxyCTX1B and 54-deoxyCTX1B in *V. louti* (15/41) are shown as an example in Fig. 4 (c–f). No CTX analogues were detected in the *C. leucas* tissues, probably due to the small concentration of CTX3C which is smaller than the LOQ for CTX3C by LC-HRMS analysis.

## 4. Discussion

In this work, a multidisciplinary characterization methodology combining CBA, immunoassay and instrumental analysis techniques are presented for the determination of the CTXs contents in fish samples from the Indian Ocean.

The toxicity values of the four flesh tissues evaluated with Neuro-2a CBA ranged between 0.01 and 2.69 μg CTX1B equiv.·kg$^{-1}$, which are equal or higher than the guidance values set by the US FDA for fish tissue (0.01 μg CTX1B equiv.·kg$^{-1}$). When evaluating the liver samples of *V. louti* (02/DIV/36), *L. bohar* (02/DIV/38) and *C. leucas* (20/10), even

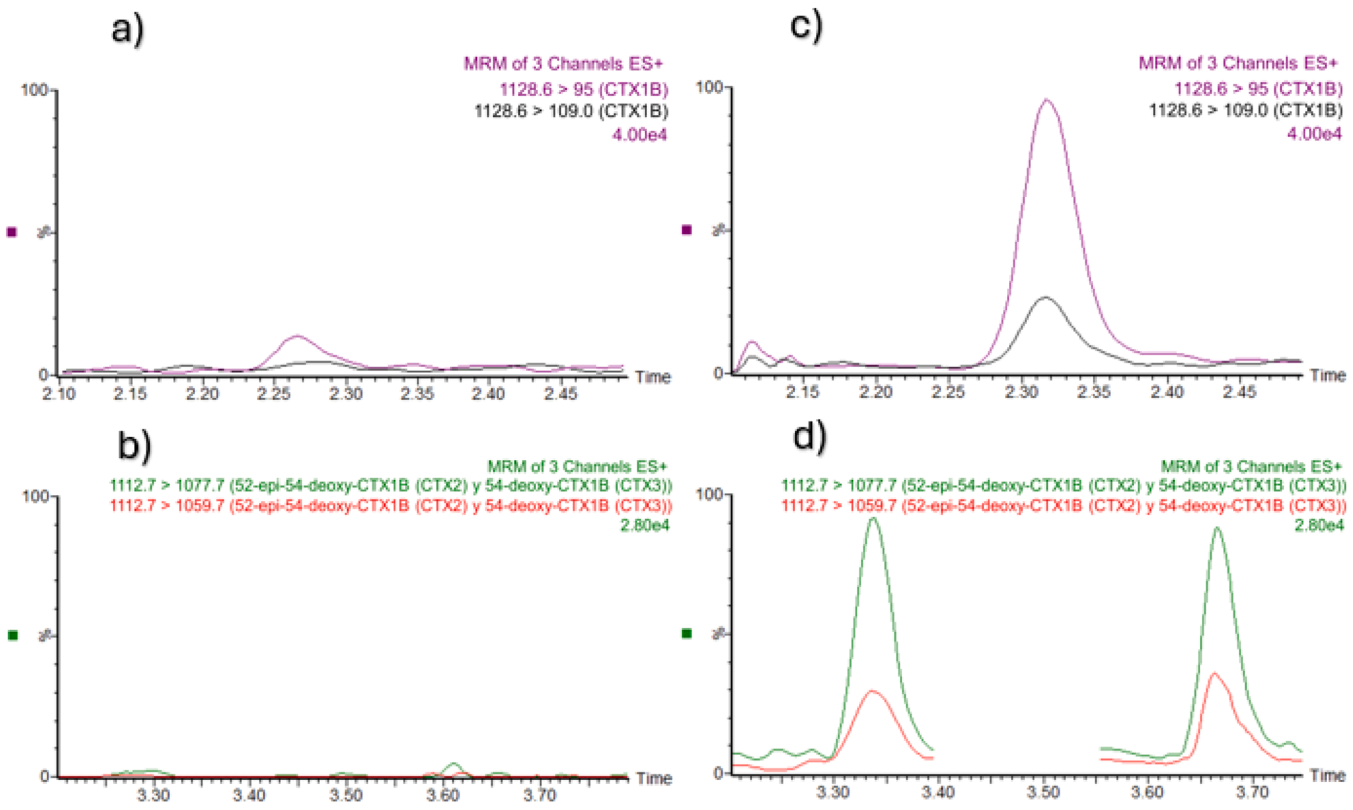


**Fig. 3.** Multiple Reaction Monitoring chromatogram of transitions monitored obtained for (a) CTX1B without SPE, (b) 52-*epi*-54-deoxyCTX1B and 54-deoxyCTX1B without SPE, (c) CTX1B after SPE and (d) 52-*epi*-54-deoxyCTX1B and 54-deoxyCTX1B after SPE of the flesh of *V. louti* (15/41) and analysis by LC-MS/MS.

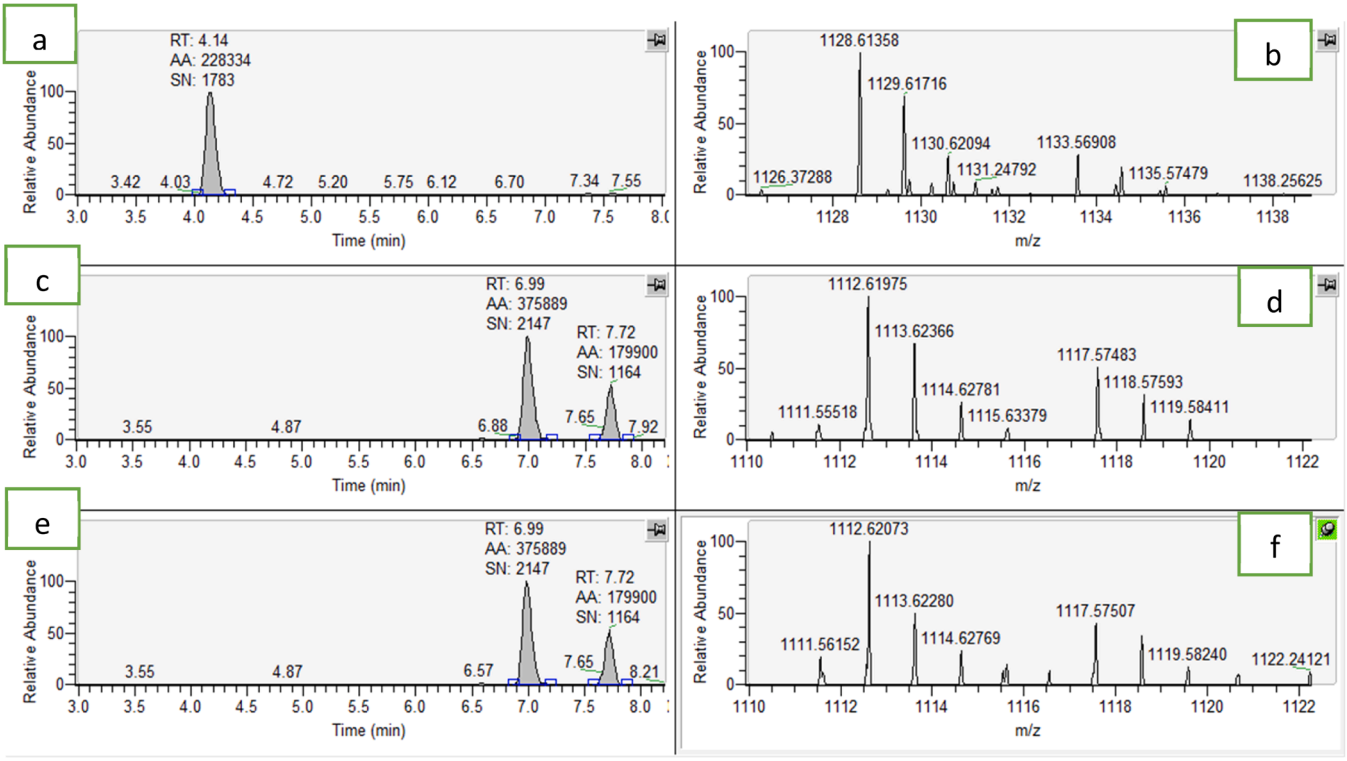


**Fig. 4.** Evidence for the presence of ciguatoxins (CTXs): (a) Extracted ion chromatogram of CTX1B $\sum$ ($[M\text{-}H_2O+H]^+ + [M+H]^+ + [M+NH_4]^+ + [M+Na]^+$) in flesh of *V. louti* (15/41) and (b) HRMS exact mass spectra of CTX1B at 4.1 min $[M+NH_4]^+$ at *m/z* 1128.6102; (c) Extracted ion chromatogram of 52-*epi*-54-deoxyCTX1B in flesh of *V. louti* (15/41) and (d) HRMS exact mass spectra of 52-*epi*-54-deoxyCTX1B at 7.0 min $[M+NH_4]^+$ at *m/z* 1112.6152; (e) Extracted ion chromatogram of 54-deoxyCTX1B in flesh of *V. louti* (15/41) *and* (f) HRMS exact mass spectra of 54-deoxyCTX1B at 7.7 min $[M+NH_4]^+$ at *m/z* 1112.6152.

higher toxicity values were observed. The liver of *C. leucas* (20/10) exhibited the highest toxicity value among the four fish (6.50 μg CTX1B equiv.·kg$^{-1}$). With regard to viscera, *V. louti* (02/DIV/36) exhibited the highest value (4.77 μg CTX1B equiv.·kg$^{-1}$). Results are in accordance with previous Neuro-2a CBA evaluations of fish from La Réunion reported by Leonardo et al. (2020), with values in flesh between 0.01 to 2.48 μg CTX1B equiv.·kg$^{-1}$. The fish from this study showed higher toxicity levels compared to fish from other regions with CP such as the Selvages Islands, where CTX-like toxicity was 0.03–0.08 μg CTX1B equiv.·kg$^{-1}$ in flesh and 0.01–0.27 μg CTX1B equiv.·kg$^{-1}$ in liver (Tudó et al., 2022). And similar toxicity levels compared to fish from La Réunion were observed, where CTX-like toxicity in fles was 0.009 - 2.481 μg CTX1B equiv.·kg$^{-1}$ $^{\text{in flesh}}$ (Leonardo et al., 2020).

In this study, *C. leucas* stands out for the large difference in CTX toxicity between flesh and liver, being 650-fold higher in the liver. For *V. louti* and *L. bohar* specimens, the CTX toxicity in the viscera was 2.97-fold higher than in the liver, and the toxicity in the liver was 2.35-fold higher than in the flesh. The higher toxicity found in liver compared to flesh is in accordance with Chan et al. (2011), where the highest CTX concentration in liver was 9-fold higher than in flesh. Ramos-Sosa et al. (2022) also stands out for the large difference between flesh and liver of black moray eels from the Canary Islands, with a ratio of 48.32-fold. However, most articles evaluate CTXs only in flesh (Estevez et al., 2019; Hamilton et al., 2002; Oshiro et al., 2010; Pottier et al., 2002; Sanchez-Henao et al., 2019). This approach may be justified by the fact that the flesh is the most consumed tissue but, in some regions and for some species the viscera are also consumed.

These same fish were also analysed by a magnetic bead-based immunoassay (Gaiani et al., 2020; Leonardo et al., 2020,). With this technique, the flesh of *V. louti* (15/41) provided the highest contents of CTX-like compounds (1.15 μg CTX1B equiv.·kg$^{-1}$). For the *V. louti* (02/DIV/36) and *L. bohar* (02/DIV/38), it was observed that the liver was the tissue with the highest CTXs contents (0.58 and 0.78 μg CTX1B equiv.·kg$^{-1}$), as also observed with Neuro-2a CBA, while in this case the flesh became the second tissue with high CTXs contents (0.31 and 0.60 μg CTX1B equiv.·kg$^{-1}$), followed by the viscera which were the ones that lower contents presented (0.16 and 0.52 μg CTX1B equiv.·kg$^{-1}$). In this case, the concentration in the liver was between 1.87-fold and 1.30-fold higher than in flesh. These values in flesh are in accordance with results from Leonardo et al. (2020) where CTX-like compounds were also detected between 0.01 to 0.15 μg CTX1B equiv.·kg$^{-1}$ in fish from La Réunion. Regarding *C. leucas* (20/10), the CTXs contents values for the three tissues were below LOD (0.001 μg CTX1B equiv.·kg$^{-1}$) for CTXs of the CTX1B series but 0.04 μg 51-hydroxyCTX3C equiv.·kg$^{-1}$ were detected in the liver. The results indicate the presence of CTX3C congeners and the absence of CTX1B analogues in the *C. leucas* (20/10). In general, the CTXs contents obtained with the immunoassay were lower than those provided by the Neuro-2a CBA, as probably expected taking into account that samples could contain CTXs not recognized by the monoclonal antibodies. It is important to highlight that no CTXs were detected (neither from the CTX1B nor CTX3C series) in the flesh and the viscera of *C. leucas* (20/10), and only little amounts in the liver. Therefore, the toxicological response obtained with CBA for these samples must also be attributed to other CTX congeners. In fact, the immunoassay and the Neuro-2a CBA have different detection principles: whereas the Neuro-2a CBA evaluates a composite toxicological response due to the effect of the family of CTXs on the voltage-gated sodium channels (VGSCs) of Neuro-2a cells, the immunoassay responds to the structural interaction between specific CTXs and their mAbs.

In all analytical techniques, matrix compounds may interfere with the assay, therefore affecting the toxin quantifications. Nevertheless, CBAs include appropriate controls to discriminate these matrix effects, and the immunoassay used in this work, which exploits the use of magnetic beads as immobilization supports and is performed in suspension, is highly robust to this type of interferences. The analysis of crude fish extracts with spectrometry methods, however, may not only interfere with the assay but also cause problems in the instrumentation. Therefore, in the present study, extracts were purified using SPE techniques with different cartridges to minimise or reduce the matrix effect of the samples. After evaluating both cleaning cartridges separately and combined, the combined use of Florisil SPE and C18 SPE presented the better recoveries and matrix effect results in the spiked *L. bohar* flesh. Afterwards, the recovery rates in spiking experiments from the combined use of Florisil SPE and C18 in the three tissues of *C. leucas* (20/10) including flesh, liver and viscera ranged from 48 % to 89 % for CTX1B, which is in agreement with the guidance document SANTE/12682/2019. Liver showed the highest matrix effect followed by the viscera and then the flesh. Matrix effect for all three tissues was very good, obtaining results between 81 % and 101 %. Our results are in accordance with previous studies such as Stewart et al. (2010) and Wu et al. (2011) that conducted spiking experiments with several fish species and obtained recoveries between 27 % and 85 % for CTX1B. Spielmeyer et al. (2021) carried out the same experiments, obtaining recoveries between 39 % and 62 %. Our values are also comparable to values reported in other studies like in Nagae et al. (2021), which reported mean recovery rates of 87 % to 107 % for CTX1B and CTX3C in snapper and grouper.

In this study, the LC-MS/MS method was optimized based on Yogi et al. (2011) and according to the characteristics of the mass spectrometer used to carry out this work. The lack of pure standards and reference materials has been a major limitation to perform the complete validation of this method. For this reason, the most relevant criteria have been selected for quantification including linearity, LOD, and LOQ and repeatability. Several potential congeners of the CTX1B-group (CTX1B, 52-*epi*-54-deoxyCTX1B, and 54-deoxyCTX1B) and CTX3C-group (CTX3C) were identified in the different tissues of the fish. The implementation of the method allowed the identification of CTX1B as the main responsible for the CP in the samples from La Réunion. The CTX1B was the only analogue present in all fishes except the shark (*C. leucas*), where only CTX3C was identified. At this point, it is important to highlight that the immunosensing tool detected CTXs from the CTX3C series in only this sample. The high specificity of the immunoassay is therefore corroborated by the LC-MS/MS confirmation.

Among the samples mentioned in Table 1, three are toxic reference material in CITEB laboratory (La Réunion), while one of the fish, *V. louti* (15/41), was involved in a CP case in 2015. This species, weighing 5.7 kg, was purchased in Mauritius, and transported to La Réunion to be consumed. Six out of seven people who ate this fish on 03/25/2015 presented symptoms characteristic of CP such as paraesthesia of the extremities and mouth, superficial dysesthesia, headache, myalgia, diarrhoea, reversal of the sensation of cold and heat, asthenia, and pruritus. The presence of CTXs in the flesh of *V. louti* confirms this episode as a ciguatera poisoning event in La Réunion.

The CTXs determined by LC-MS/MS were confirmed and characterized using LC-HRMS thanks to the measured *m/z*, mass accuracy (ppm), ring double bond equivalents (RDBE) and the mono-isotopic pattern (M+1 ion). Quantifications with and without sample clean-up were performed, finding LC-HRMS quantifications lower with clean-up except for the liver and viscera of *V. louti* (02/DIV/36), but an improvement of the mass accuracy was achieved (< 4.0 ppm). The presence of CTX1B was confirmed by LC-HRMS in all fishes except the shark (*C. leucas*), where no CTXs were detected. Both LC-MS analyses indicate that the *V. louti* flesh (15/41) showed the highest CTX1B concentration, followed by the *L. bohar* flesh (02/DIV/38) and *V. louti* flesh (02/DIV/36). Another two CTX1B congeners in the *V. louti* flesh (15/41) and in the *V. louti* (02/DIV/36) flesh were also confirmed by LC-HRMS. CTX1B was detected at a very low concentration in the *L. bohar* flesh (02/DIV/38), this was not identified in the previous analyses by LC-MS/MS. This could be explained by a possible matrix compound of the viscera tissue which interfere in the LC-MS/MS and not interfere in the LC-HRMS analyses. In general, concentrations were higher than those obtained by LC-MS/MS, but this could be due to the sum of four adducts $\sum$ $[M+H\text{-}H_2O]^+$ + $[M+H]^+$ + $[M+NH_4]^+$ + $[M+Na]^+$ used for the quantification. A good

correlation was found between the quantifications (sum of four adducts) performed by LC-MS/MS and LC-HRMS (Figure S4) after the SPE clean-up, as both methodologies are based on the same chemical structure recognition.

In accordance with the results of the toxin profile from *L. bohar (*02/DIV/38) and *V. louti* species (02/DIV/36 and 15/41) of this study, Oshiro et al. (2021) detected CTX1B-type and CTX3C-type toxins in *V. louti* (two specimens), *Lutjanus fulviflamma* (two specimens), and *Gymnothorax javanicus* (one specimen). Additionally, the *V. louti* specimens obtained from local market or fishermen in Fiji (Sigatoka and Suva) in 2014-2015 contained three CTX1B-type analogues. In the *L. fulviflamma*, CTX1B-type toxins were not detected but trace amounts of CTX3C and 49-*epi*CTX3C were detected in the two specimens obtained from Nadi. The *Gymnothorax javanicus* purchased in Lautoka contained only CTX3C-type toxins (Oshiro et al., 2021). Afterwards, in 2022, Oshiro et al. have used LC-MS/MS, to analyse CTX in the flesh of 154 individuals from various locations, detecting CTX in 99 samples (64 %). In 65 fish (43 %), CTX levels exceeded the US FDA guidance level (0.01 μg equiv. CTX1B·kg$^{-1}$). Additionally, although the highest total CTX level was 0.38 μg kg$^{-1}$, the consumption of 180 g of this specimen was assumed to cause CP. Moreover, only CTX1B, 52-*epi*-54-deoxyCTX1B, and 54-deoxyCTX1B were detected, with the relative contribution of the three CTX1B analogues to the total toxin content being 35 ± 7.7 %, 27 ± 8.1 %, and 38 ± 5.6 %, respectively. In our study, the analogues from the CTX1B-type have been the major contributor to the total toxin content of the fish from La Réunion. These results are aligned with the results from Fiji (Sigatoka and Suva) that detected CTX1B (0.08 and 0.03 μg kg$^{-1}$, respectively), 52-*epi*-54-deoxyCTX1B (0.06 and 0.02 μg kg$^{-1}$, respectively), and 54-deoxyCTX1B (0.10 and 0.03 μg kg$^{-1}$, respectively) (Oshiro et al., 2021). However, the toxin profile described in our study and Oshiro et al. (2022) is not the same as the one described in Spielmeyer et al. (2022) where 2,3-dihydroxyCTX3C (38 %) and its 49-epimer (36 %) was the dominant congener in most samples. This could be explained by the different *Gambierdiscus* species among different areas. On the other side, the result of this study supports one of the conclusions from Spielmeyer et al, 2022 describing that the toxin profile of samples from the Pacific Ocean and the Indian Ocean showed no differences concerning detected congeners.

When comparing the CTX contents obtained from the CBA with the results provided by LC-MS/MS and LC-HRMS, it is important to take into account that these instrumental analysis techniques determine individual CTX analogue contents based on their chemical structures and not their toxicities. Therefore, discrepancies may arise due to other CTX congeners not detected because of the low contents and/or because they are not targeted by the LC-MS methods. Nevertheless, flesh presented higher correspondence between the techniques in comparison to liver and viscera, probably due to the lower matrix effects observed. At this point it is necessary to emphasise the importance of matrix effect assessment and the importance of quantification under non-matrix effect conditions (or, where this is not possible, the application of correction factors) in order to provide more accurate toxin quantifications. Apart from the no targeted compounds and matrix effects, LC-MS/MS methods are not optimized for all described CTX congeners as no standard material is available. We cannot rule out the presence of other ciguatoxins that would justify the great toxic response that exists in CBA, i.e *C. leucas* liver. Research efforts are now being focused on finding other molecules responsible for this toxicity, but this research is beyond this work.

When the LC-MS/MS and LC-HRMS results are compared to the ones obtained with the magnetic bead-based immunoassay, almost all samples correlate very well, which is not surprising considering that, in contrast with CBA, instrumental analysis techniques and immunoassays are both structural approaches. It is important to note that the immunoassay recognizes 54-deoxyCTX1B very well, but it barely recognizes the 52-*epi*-54-deoxyCTX1B, which was detected by LC-MS/MS and LC-HRMS in two samples (Tsumuraya and Hirama, 2025).

Vernoux et al. (1985) reported that distribution of CTXs in liver versus flesh varies significantly within and between fish species. This is consistent with our results where the liver of *V. louti* (02/DIV/36) and *C. leucas* (20/10) presents higher toxicity than flesh, however the amount of CTX1B and CTX3C detected by LC-MS/MS and LC-HRMS does not correspond to the total toxicity of the sample, suggesting that the toxicity found in the liver may be explained by other CTX congeners. Further research is being conducted in order to determine unknown compounds responsible for CTX-like toxicity in fish from La Réunion. Addressing evaluation of toxins in the liver and viscera in addition to flesh gives further knowledge for a better environmental assessment in the ecosystems.

In this study, the implementation of four complementary techniques (a Neuro-2a CBA for the detection of CTX-like toxicity, an immunoassay for the detection of CTX-like compounds from the CTX1B and CTX3C series, and LC-MS/MS and LC-HRMS for the detection and confirmation of CTX1B and different analogues of CTX1B-group and CTX3-group) is a very useful strategy to quantify CTXs in fishes and assess the risk they may present for consumers. Further studies are being carried out to implement the knowledge about the incidence of CP in these areas and to relate their incidence with environmental conditions, among others.

## 5. Conclusions

Herein, we provide for the first time the toxin profile of fishes from La Réunion, thereby demonstrating the potential hazard for CP in this area. Fish (flesh, liver, and viscera) extracts were initially analysed by Neuro-2a CBA and a magnetic bead-based immunoassay as screening methods of CTXs contents and subsequently analysed by LC-MS/MS and LC-HRMS. Although the toxin profiles of fish extracts without clean-up were obtained, high matrix effects were observed and therefore a clean-up step was implemented.

The LC-MS/MS methodology was optimized, and the mass spectrometry methods were updated according to the newest literature data to include the maximum number of CTX analogues. The optimization of the clean-up protocol was performed in the three matrices (flesh, liver, and viscera) by LC-MS/MS. A study was conducted with the evaluation of matrix effect and recoveries of CTX1B, obtaining good results. The combination of C18 and Florisil cartridges showed the best results.

Three analogues of the CTX4A-group (CTX1B, 52-*epi*-54-deoxyCTX1B and 54-deoxyCTX1B) and one analogue of CTX3C-group were identified as the main congeners present in the samples. Six samples, including flesh, liver, and viscera, showed CTX1B in their toxin profile by LC-MS/MS and seven samples by LC-HRMS, which could be due the low concentration detected together with a possible higher matrix effect of the viscera in the LC-MS/MS system. *C. leucas* (20/10) was the only fish that did not show CTX1B in any of the three tissues analysed but showed CTX3C in the liver. We cannot rule out the possibility of presence of I-CTXs in these samples. Although on analyses did not result in any I-CTX detected, further work should be devoted to improve the clean-up and analytical protocol for this type of CTXs. Further research should be done on these species from this area, increasing the number of fish specimens. All signals detected by LC-MS/MS were confirmed by LC-HRMS, being CTX1B the main compound detected and confirmed. LC-HRMS is a very useful approach that combines qualitative and quantitative analyses. The combination of high resolution, mass accuracy and restrictive criteria was crucial for identifying targeted CTX compounds.

In this study, the maximum difference in CTX-like toxicity between liver and flesh was 650-fold greater in liver than in flesh and the maximum difference between viscera and liver was 2.97-fold greater in viscera. CTX compounds were confirmed also in the liver, showing that the liver can contribute as an early warning strategy and/or environmental CP indicator to describe better the transfer and biotransformation of CTX in the food web in a specific area. The multi-disciplinary approach used in this study has proved to be effective for a better characterization of the incidence of CP.

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## Appendix A. Supplementary data

Figure S1. Concentration-response curves of Neuro-2a CBA to CTX1B or *C. leucas* extract, in the absence (O/V -) and presence (O/V+) of Ouabain and Veratridine: (a) Concentration-response curve of Neuro-2a cells after exposure to CTX1B standard; Dose-response curve of Neuro-2a cells after the exposure to (b) flesh *C. leucas* extract, (c) liver *C. leucas* extract and (d) viscera *C. leucas* extract. Data represent the average and the standard deviation (SD) for three replicates on the same day of experimentation. Figure S2: Optimization of the clean-up using flesh of *L. bohar* specimen through the different strategies, including no SPE, Florisil individually, C18 individually and the combination of Florisil and C18. Recovery and Matrix effect are expressed as Recov (%) and SI (%), respectively; Figure S3: Recovery (Recov, %) and Matrix effect (SI, %) for C. leucas matrices (flesh, liver and viscera) with the combination of Florisil and C18 cartridges; Figure S4: Correlations between CTXs contents (μg·kg $^{-1}$) obtained using the LC-MS/MS and LC- HRMS. Table S1. Compound specific LC-MS/MS parameters used for the analysis of selected ciguatoxins. Note: (*) Transition used for quantification; *Table S2.* Compound specific LC-MS/MS parameters used for the CTX4A-group*; Table S3.* Compound specific LC-MS/MS parameters used for the CTX3C-group; *Table S4.* Compound specific LC-MS/MS parameters used for the C-CTX1-group; and *Table S5.* Compound specific LC-MS/MS parameters used for the I-CTX1-group.

## CRediT authorship contribution statement

**Lourdes Barreiro-Crespo:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Conceptualization. **Andres Sanchez-Henao:** Methodology, Formal analysis. **Sandra Gimeno-Monforte:** Methodology, Formal analysis. **Jaume Reverté:** Methodology, Formal analysis. **Mònica Campàs:** Writing – review & editing. **Takeshi Tsumuraya:** Writing – review & editing. **Jorge Diogène:** Writing – review & editing, Supervision, Conceptualization. **Alina Tunin-Ley:** Formal analysis. **Fanny Maillot:** Formal analysis. **Cintia Flores:** Writing – review & editing, Formal analysis. **Núria Fontanals:** Writing – review & editing. **Francesc Borrull:** Writing – review & editing. **Jean Turquet:** Writing – review & editing, Supervision, Investigation, Conceptualization. **Maria Rambla-Alegre:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Conceptualization.

## 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.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.hal.2025.102882.

## Data availability

Data will be made available on request.

## References

Abraham, A., Jester, E.L.E., Granade, H.R., Plakas, S.M., Dickey, R.W., 2012. Caribbean ciguatoxin profile in raw and cooked fish implicated in ciguatera. Food Chem. 131 (1), 192–198.

Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail (ANSES): Contamination des requins, notamment tigre et bouledogue, par des ciguatoxines: occurrence, méthodes analytiques, cas humains rapportés et éléments d'éthologie. Avis de l'Anses Rapport d'expertise collective. Avis de l'Anses Saisine n° 2013-SA-0198 (revised edition, january 2015). https://www.anses.fr/en/system/files/ERCA2013sa0198EN.pdf.

Bagnis, R., Kuberski, T., Laugier, S., 1979. Clinical observations on 3009 cases of ciguatera (fish poisoning) in the South Pacific. Am. J. Trop. Med. Hyg. 28, 1067–1073.

Bagnis, R., Chanteau, S., Chungue, E., Hurtel, J.M., Yasumoto, T., Inoue, A., 1980. Origins of ciguatera fish poisoning: a new dinoflagellate, gambierdiscus toxicus Adachi and Fukuyoa, definitively involved as a causal agent. Toxicon 18 (2), 199–208.

Biais, G., Taquet, M., 1992. La pê che locale aux abords de la Réunion (Editions de l'Ifremer). Available at: https://archimer.ifremer.fr/doc/00000/1455/.

Bleeker, P., 1874. Mémoire sur les sciénoïdes et les sillaginoïdes de l'Inde archipélagique. *Verslagen en Mededeelingen der Koninklijke Akademie van Wetenschappen.* Afd. Natuurkd. 14, 1–76.

Boisier, P., Ranaivoson, G., Rasolofonirina, N., Andriamahefazafy, B., Roux, J., Chanteau, S., Satake, M., Yasumoto, T., 1995. Fatal mass poisoning in Madagascar following ingestion of a shark (Carcharhinus leucas): clinical and epidemiological aspects and isolation of toxins. Toxicon: Off. J. Int. Soc. Toxinology 33 (10), 1359–1364.

Caillaud, A., Yasumoto, T., Diogene, J., 2010. Detection and quantification of maitotoxin-like compounds using a neuroblastoma (Neuro-2a) cell-based assay. Application to the screening of maitotoxin-like compounds in gambierdiscus spp. Toxicon 56, 36–44.

Caillaud, A., Eixarch, H., de la Iglesia, P., Rodríguez, M., Domínguez, L., Andree, K.B., Diogène, J., 2012. Towards the standardisation of the neuroblastoma (Neuro-2a) cell-based assay for ciguatoxin-like toxicity detection in fish: application to fish caught in the Canary Islands. Food Addit. Contam. Chem. Anal. Control Expo. Risk Assess. 29 (6), 1000–1010.

Chan, W.H., Mak, Y.L., Wu, J.J., Jin, L., Sit, W.H., Lam, J.C.W., de Mitcheson, Y.S., Chan, L.L., Lam, P.K.S., Murphy, M.B., 2011. Spatial distribution of ciguateric fish in the Republic of Kiribati. Chemosphere 84, 117–123.

Chinain, M., Gatti, C.M., Ung, A., Cruchet, P., Revel, T., Viallon, J., Sibat, M., Varney, P., Laurent, V., Hess, P., 2020. Evidence for the range expansion of ciguatera in French Polynesia: A revisit of the 2009 mass-poisoning outbreak in Rapa Island (Australes Archipelago). Toxins 12, 759.

Darius, H.T., Ponton, D., Revel, T., Cruchet, P., Ung, A., Fouc, M.T., Chinain, M., 2007. Ciguatera risk assessment in two toxic sites of French Polynesia using the receptorbinding assay. Toxicon 50 (5), 612–626.

Dechraoui, M.Y.B., Tiedeken, J.A., Persad, R., Wang, Z., Granade, H.R., Dickey, R.W., Ramsdell, J.S., 2005. Use of two detection methods to discriminate ciguatoxins from brevetoxins: application to great barracuda from Florida Keys. Toxicon 46 (3), 261–270.

Dickey, R.W., Plakas, S.M., 2010. Ciguatera: A public health perspective. Toxicon 56, 123–136.

Diogène, J., Reverté, L., Rambla-Alegre, M., del Río, V., de la Iglesia, P., Campàs, M., Palacios, O., Flores, C., Caixach, J., Ralijaona, C., et al., 2017. Identification of ciguatoxins in a shark involved in a fatal food poisoning in the Indian Ocean. Sci. Rep. 7, 8240.

Document N° SANTE/2021/808, 2021. Commission Implementing Regulation (EU) 2021/808 of on the performance of analytical methods for residues of pharmacologically active substances used in food-producing animals and on the interpretation of results as well as on the methods. Off. J. Eur. Communities 180. L180/84-L180/109.

Document N° SANTE/12682/2019, 2020. Analytical quality control and method validation procedures for pesticide residues analysis in food and feed; implemented by 1 january 2020. European Union: Brussels, Belgium aqcguidance_sante_2019_12682.pdf (eurl-pesticides.eu.

Eschmeyer, W.N., Fricke, R., 2022. Cat. Fishes Electron. Version. Available at https://researcharchive.calacademy.org/research/ichthyology/catalog/SpeciesByFamily.asp.

Estevez, P., Castro, D., Manuel Leao, J., Yasumoto, T., Dickey, R., Gago-Martinez, A., 2019. Implementation of liquid chromatography tandem mass spectrometry for the analysis of ciguatera fish poisoning in contaminated fish samples from Atlantic coasts. Food Chem. 280, 8–14.

Estevez, P., Oses Prieto, J., Burlingame, A., Gago Martinez, A., 2023. Characterization of the ciguatoxin profile in fish samples from the Eastern Atlantic Ocean using capillary liquid chromatography-high resolution mass spectrometry. Food Chem. Aug; 418, 135960. https://doi.org/10.1016/j.foodchem.2023.135960. PMID36965390.

Estevez, P., Oses-Prieto, J., Castro, D., Penin, A., Burlingame, A., Gago-Martinez, A., 2024. First detection of algal Caribbean ciguatoxin in amberjack causing ciguatera poisoning in the Canary Islands (Spain). Toxins 16, 189.

FAO, W.H.O., 2020. Report of the expert meeting on ciguatera poisoning. Rome, 19–23 November 2018 Food Safety and Quality. Rome: World Health Organ. (9). https://doi.org/10.4060/ca8817en.

FAO, 2020. The State of World Fisheries and Aquaculture 2020. Sustainability in action. FAO, Rome.

Friedman, M.A., Fernandez, M., Backer, L.C., Dickey, R.W., Bernstein, J., Schrank, K., Kibler, S., Stephan, W., Gribble, M.O., Bienfang, P., Bowen, R.E., Degrasse, S., Flores Quintana, H.A., Loeffler, C.R., Weisman, R., Blythe, D., Berdalet, E., Ayyar, R., Clarkson-Townsend, D., Swajian, K., Benner, R., Brewer, T., Fleming, L.E., 2017. An updated review of Ciguatera fish poisoning: clinical, epidemiological, environmental, and public health management. Mar. Drugs 15 (3), 72.

Frisch, A.J., Cameron, D.S., Pratchett, M.S., Williamson, D.H., Williams, A.S., Reynolds, A.D., et al., 2016. Key aspects of the biology, fisheries and management of coral grouper. Rev. Fish Biol. Fish. 26, 303–325. https://doi.org/10.1007/s11160-016-9427-0.

Gaiani, G., Leonardo, S., Tudó, À., Toldrà, A., Rey, M., Andree, KB., Tsumuraya, T., Hirama, M., Diogène, J., O'Sullivan, CK., Alcaraz, C., Campàs, M., 2020. Rapid detection of ciguatoxins in Gambierdiscus and Fukuyoa with immunosensing tools. Ecotoxicol Env. Saf. Nov. 204, 111004. https://doi.org/10.1016/j.ecoenv.2020.111004. Epub 2020 Aug 5. PMID: 32768745.

Habermehl, G.G., Krebs, H.C., Rasoanaivo, P., Ramialiharisoa, A., 1994. Severe CPin madagascar - a case - report. Toxicon 32, 1539–1542.

Halstead, B.W., Cox, K.W., 1973. An investigationg on fish poisoning in Mauritius. Proc. R. Soc. Arts. Sci. Mautitius 4, 1–26.

Hamilton, B., Hurbungs, M., Vernoux, J.-P., Jones, A., Lewis, R.J., 2002a. Isolation and characterisation of Indian Ocean ciguatoxin. Toxicon 40 (6), 685–693. https://doi.org/10.1016/S0041-0101(01)00259-8.

Hamilton, B., Hurbungs, M., Jones, A., Lewis, R.J., 2002b. Multiple ciguatoxins present in Indian Ocean reef fish. Toxicon 40 (9), 1347–1353.

Hardison, D.R., Holland, W.C., McCall, J.R., Bourdelais, A.J., Baden, D.G., Darius, H.T., Chinain, M., Tester, P.A., Shea, D., Quintana, H.A.F., 2016. Fluorescent receptor binding assay for detecting ciguatoxins in fish. PLoS One 11 (4), e0153348.

Heemstra, P.C., Randall, J.E., 1993. FAO species catalogue. Vol. 16. groupers of the world (family serranidae, subfamily epinephelinae). An Annotated and illustrated catalogue of the grouper, rockcod, hind, coral grouper and lyretail species known to date. Rome: FAO. FAO Fish. Synop. 125 (16), 382. https://doi.org/10.1016/j.rsma.2020.101388.

Holmes, M.J., Lewis, R.J., Poli, M.A., Gillespie, N.C., 1991. Strain dependent production of ciguatoxin precursors (gambiertoxins) by Gambierdiscus toxicus (Dinophyceae) in culture. Toxicon 29, 761–775.

Ikehara, T., Kuniyoshi, K., Oshiro, N., Yasumoto, T., 2017. Biooxidation of ciguatoxins leads to species-specific toxin profiles. Toxins (Basel) 9, 205.

Kryuchkov, F., Robertson, A., Miles, C.O., Mudge, E.M., Uhlig, S., 2020. LC–HRMS and chemical derivatization strategies for the structure elucidation of Caribbean ciguatoxins: identification of c-CTX-3 and -4. Mar. Drugs 18, 182. https://doi.org/10.3390/md18040182.

Laza-Martínez, A., David, H., Riobo, P., Miguel, I., Orive, E. J.,2016. Eukaryotic microbiol. 63, 481−497.

Le Manach, F., Bach, P., Barret, L., Guyomard, D., Fleury, P.G., Sabarros, P.S., et al., 2015. Reconstruction of the domestic and distant water fisheries catch of la réunion (France), 1950–2010," in fisheries catch reconstructions in the Western Indian Ocean 1950–2010. In: Manach, F., Pauly, D. (Eds.), Reconstruction of the domestic and distant water fisheries catch of la réunion (France), 1950–2010," in fisheries catch reconstructions in the Western Indian Ocean 1950–2010. Fish. Cent. Univ. B. C.: Fish. Cent. Res. Rep. 23, 83–98.

Lewis, R.J., Vernoux, J.P., Brereton, I.M., 1998. Structure of Caribbean ciguatoxin isolated from Caranx latus. J. Am, Chem, Soc 120, 5914–5920.

Lewis, R.J., Yang, A., Jones, A., 2009. Rapid extraction combined with LC-tandem mass spectrometry (CREM-LC/MS/MS) for the determination of ciguatoxins in ciguateric fish flesh. Toxicon 54 (1), 62–66.

Leonardo, S., Gaiani, G., Tsumuraya, T., Hirama, M., Turquet, J., Sagristà, N., Rambla-Alegre, M., Flores, C., Caixach, J., Diogène, J., O'Sullivan, CK., Alcaraz, C., Campàs, M., 2020. Addressing the analytical challenges for the detection of ciguatoxins using an electrochemical biosensor. Anal Chem. Apr 7 (7), 4858–4865. https://doi.org/10.1021/acs.analchem.9b04499, 92Epub 2020 Mar 17. PMID: 32133843.

Lewis, R.J., Inserra, M., Vetter, I., Holland, W.C., Hardison, D.R., Tester, P.A., Litaker, R. W., 2016. Rapid extraction and identification of maitotoxin and ciguatoxin-like toxins from Caribbean and Pacific gambierdiscus using a new functional bioassay. PloS One 11 (7), e0160006. https://doi.org/10.1371/journal.pone.0160006.

Litaker, R., Holland, W., Hardison, D., Pisapia, F., Hess, P., Kibler, S., Tester, P., 2017. Ciguatoxicity of gambierdiscus and Fukuyoa species from the Caribbean and Gulf of Mexico. PLoS One 12, e185776.

Loeffler, C.R., Spielmeyer, A., Blaschke, V., Bodi, D., Kappenstein, O., 2023. Ciguatera poisoning in Europe: A traceback to Indian Ocean sourced snapper fish (Lutjanus bohar). Food Control 151, 109799. https://doi.org/10.1016/j.foodcont.2023.109799.

Mahé, K., Gentil, C., Brisset, B., Evano, H., Lepetit, C., Boymond-Morales, R., Telliez, S., Dussuel, A., Rungassamy, T., Elleboode, R., MacKenzie, K., Roos, D., 2022. Biology of exploited groupers (Epinephelidae family) around La Réunion Island (Indian Ocean). Front. Mar. Sci. 9, 935285. https://doi.org/10.3389/fmars.2022.935285.

Mak, Y.L., Wai, T.C., Murphy, M.B., Chan, W.H., Wu, J.J., Lam, J.C., Chan, L.L., Lam, P. K., 2013. Pacific ciguatoxins in food web components of coral reef systems in the Republic of Kiribati. Env. Sci. Technol. 47 (24), 14070–14079. https://doi.org/10.1021/es403175d.

Manger, R.L., Leja, L.S., Lee, S.Y., Hungerford, J.M., Wekell, M.M., 1993. Tetrazolium-based cell bioassay for neurotoxins active on voltage-sensitive sodium channels: semiautomated assay for saxitoxins, brevetoxins, and ciguatoxins. Anal. Biochem. 214 (1), 190–194. https://doi.org/10.1006/abio.1993.1476.

Mudge, E.M., Miles, C.O., Ivanova, L., Uhlig, S., James, K.S., Erdner, D.L., Fæste, C.K., McCarron, P., Robertson, A., 2023. Algal ciguatoxin identified as source of ciguatera poisoning in the Caribbean. Chemosphere 330, 138659. https://doi.org/10.1016/j.chemosphere.2023.138659.

Mudge, E.M., Robertson, A., Uhlig, S., McCarron, P., Miles, C.O., 2024. 3-Epimers of Caribbean ciguatoxins in fish and algae. Toxicon: off. J. Int. Soc. Toxinology 237, 107536. https://doi.org/10.1016/j.toxicon.2023.107536.

Murata, M., Legrand, A.M., Ishibashi, Y., Fukui, M., Yasumoto, T., 1990. Structures and configurations of ciguatoxin from the moray eel Gymnothorax javanicus and its likely precursor from the dinoflagellate Gambierdiscus toxicus. J. Am. Chem. Soc. 112, 4380–4386. https://doi.org/10.1021/ja00167a040.

Murray, J.S., Boundy, M.J., Selwood, A.I., Harwood, D.T., 2018. Development of an LC–MS/MS method to simultaneously monitor maitotoxins and selected ciguatoxins in algal cultures and P-CTX-1B in fish. Harmful Algae 80, 80–87.

Murray, J.S., Passfield, E.M.F., Rhodes, L.L., Puddick, J., Finch, S.C., Smith, K.F., van Ginkel, R., Mudge, E.M., Nishimura, T., Funaki, H., Adach, iM., Prinsep, M.R., Harwood, D.T, 2024. Targeted metabolite fingerprints of thirteen gambierdiscus, five Coolia and two Fukuyoa species. Mar. Drugs. Mar. 2 (3), 119, 22.

Nagae, M., Igarashi, T., Mizukoshi, K., Yasumoto, T., Kuniyoshi, K., Oshiro, N., 2021. Development and validation of an LC-MS/MS method for the ultra-trace analysis of Pacific ciguatoxins in fish. J. AOAC Int. 104 (5), 1272–1281. https://doi.org/10.1093/jaoacint/qsab052. September-October 2021.

Oguri, H., Hirama, M., Tsumuraya, T., Fujii, I., Maruyama, M., Uehara, H., Nagumo, Y., 2003. Synthesis-based approach toward direct sandwich immunoassay for ciguatoxin CTX3C. J. Am. Chem. Soc. 125 (25), 7608–7612. https://doi.org/10.1021/ja034990a.

Oshiro, N., Yogi, K., Asato, S., Sasaki, T., Tamanaha, K., Hirama, M., Yasumoto, T., Inafuku, Y., 2010. Ciguatera incidence and fish toxicity in Okinawa, Japan. Toxicon. 56 (5), 656–661.

Oshiro, N., Tomikawa, T., Kuniyoshi, K., Ishikawa, A., Toyofuku, H., Kojima, T., Asakura, H., 2021. LC–MS/MS analysis of ciguatoxins revealing the regional and species distinction of fish in the tropical Western Pacific. J. Mar. Sci. Eng. 9, 299. https://doi.org/10.3390/jmse9030299.

Oshiro, N., Nagasawa, H., Watanabe, M., Nishimura, M., Kuniyoshi, K., Kobayashi, N., Sugita-Konishi, Y., Asakura, H., Tachihara, K., Yasumoto, T., 2022. An extensive survey of ciguatoxins on grouper *Variola louti* from the Ryukyu Islands, Japan, using liquid chromatography–Tandem mass spectrometry (LC-MS/MS). J. Mar. Sci. Eng. 10, 423. https://doi.org/10.3390/jmse10030423.

Pearn, J.H., 2001. Neurology of ciguatera. J. Neurol. Neurosurg. Psychiatry 70, 408.

Pisapia, F., Holland, W.C., Hardison, D.R., Litaker, R.W., Fraga, S., Nishimura, T., Adachi, M., Nguyen-Ngoc, L., Séchet, V., Amzil, Z., 2017. Toxicity screening of 13 Gambierdiscus strains using neuro-2a and erythrocyte lysis bioassays. Harmful Algae 63, 173–183.

Pottier, I., Vernoux, J.P., Jones, A., Lewis, R.J., 2002. Analysis of toxin profiles in three different fish species causing ciguatera fish poisoning in Guadeloupe; French West Indies. Food Addit. Contam. 19, 1034–1042.

Quod, J.P, Turquet, J., 1994. Ciguatera in Réunion island (SW Indian Ocean): epidemiology and clinical patterns. Toxicon 34 (7), 779–785.

Quod, J.P., Bourdeau, P., Turquet, J., Guignard, A., 1994. La Ciguatera dans le DOM-TOM : aspects epidemiologiques et patheophysiologiques. Rec. Mèd. Vèt 141–146, 1702/3.

Rabenjarison, F., Ramarolahy, A.R.N., Velomora, A., Rahajaniaina, M.P., Raveloson, N. E., 2016. Intoxication à la ciguatoxine après consommation de requin à Fenerive-Est: profil épidémio-clinique et résultats de laboratoire. Ciguatera poisoning after consumption of shark in Fenerive-Est: epidemiological and clinical aspects and laboratory results. Revue d'Anesthésie-réanimation. Médecine d'Urgence et Toxicologie 8 (1), 9–12.

Ragelis, E.P., 1984. Ciguatera seafood poisoning. Seafood Toxins. (ACS Symp. Ser 262). American Chemical Society, Washington, DC, pp. 25–36.

Ramos-Sosa, M.J., García-Álvarez, N., Sanchez-Henao, A., Silva Sergent, F., Padilla, D., Estévez, P., et al., 2022. Ciguatoxin detection in flesh and liver of relevant fish species from the Canary Islands. Toxins 14, 46. https://doi.org/10.3390/toxins14010046.

Roeder, K., Erler, K., Kibler, S., Tester, P., Van The, H., Nguyen-Ngoc, L., et al., 2010. Characteristic profiles of ciguatera toxins in different strains of Gambierdiscus spp. Toxicon 56, 731–738. https://doi.org/10.1016/j.toxicon.2009.07.039.

Roos, D., Tessier, E., Guyomard, D., 1998. Evolution de l'activité halieutique à la réunion de 1990 à 1996 (Le Port (France: Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER). DRV/RH/RST/98–14.

Sadovy de Mitcheson, Y.S., Craig, M.T., Bertoncini, A.A., Carpenter, K.E., Cheung, W.W. L., Choat, J.H., et al., 2013. Fishing groupers towards extinction: a global assessment of threats and extinction risks in a billion-dollar fishery. Fish. Fish 14, 119–136. https://doi.org/10.1111/j.1467-2979.2011, 00455.x.

Sanchez-Henao, J.A., García-Álvarez, N., Fernández, A., Saavedra, P., Silva Sergent, F., Padilla, D., Acosta-Hernández, B., Martel Suárez, M., Diogène, J., Real, F., 2019. Predictive score and probability of CTX-like toxicity in fish samples from the official control of ciguatera in the Canary Islands. Sci, Total Env. 673, 576–584.

Satake, M., Murata, M., Yasumoto, T., 1993. The structure of CTX3C; a ciguatoxin congener isolated from cultured Gambierdiscus toxicus. Tetrahedron Lett. 34, 1975–1978. https://doi.org/10.1016/S0040-4039(00)91978-6.

Satake, M., Fukui, M., Legrand, A.-M., Cruchet, P., Yasumoto, T., 1998. Isolation and structures of new ciguatoxin analogs, 2,3-dihydroxyCTX3C and 51- hydroxyCTX3C, accumulated in tropical reef fish. Tetrahedron Lett. 39, 1197–1198. https://doi.org/10.1016/S0040-4039(97)10808-5.

Scheuer, P.J., Takahashi, W., Tsutsumi, J., Yoshida, T., 1967. Ciguatoxin: isolation and chemical nature. Science 155 (3767), 1267–1268.

Soliño, L., Widgy, S., Pautonnier, A., Turquet, J., Loeffler, C.R., Flores Quintana, H.A., Diogène, J., 2015. Prevalence of ciguatoxins in lionfish (Pterois spp.) from Guadeloupe, Saint Martin, and Saint Barthélmy Islands (Caribbean). Toxicon 102, 62–68.

Spielmeyer, A., Loeffler, CR., Bodi, D., 2021. Extraction and LC-MS/MS/MS analysis of ciguatoxins: A semi-targeted approach designed for fish of unknown origin. Toxins (Basel). Sep 8 (9), 630. https://doi.org/10.3390/toxins13090630, 13PMID: 34564634; PMCID: PMC8473320.

Spielmeyer, A., Loeffler, C., Kappenstein, O., 2022. Identical ciguatoxin-3C group profiles in lutjanus bohar from the Pacific and Indian Oceans - indicating the need to re-evaluate geographical CTX classifications. Front. Mar. Sci. 9, 937438. Mar. Biogeochem. 9. https://doi.org/10.3389/fmars.2022.937438.

Stewart, I., Eaglesham, G.K., Poole, S., Graham, G., Paulo, C., Wickramasinghe, W., Sadler, R., Shaw, G.R., 2010. Establishing a public health analytical service based on chemical methods for detecting and quantifying Pacific ciguatoxin in fish samples. Toxicon 56, 804–812.

Stump, K., Sherman, K., Dahlgren, C., 2017. Nassau grouper migration patterns during full moon suggest collapsed historic fish spawning aggregation and evidence of an undocumented aggregation. Bull Mar Sci 93 (2), 375–389. https://doi.org/10.5343/bms.2016.1042.

Swainson, W., 1839. On the natural history and classification of fishes, amphibians, and reptiles, or monocardian animals, 2. Spottiswoode and Co, London, p. 448.

Tester, PA., Feldman, Nau, RL., Kibler, AW., Wayne, SR., Litaker, R., 2010. Ciguatera fish poisoning and sea surface temperatures in the Caribbean Sea and the West Indies. Toxicon 56 (5), 698–710. https://doi.org/10.1016/j.toxicon.2010.02.026. OctEpub 2010 Mar 3. PMID: 20206196.

Tudó, À., Rambla-Alegre, M., Flores, C., Sagristà, N., Aguayo, P., Reverté, L., Campàs, M., Gouveia, N., Santos, C., Andree, K.B., Marques, A., Caixach, J., Diogène, J., 2022. Identification of new CTX analogues in fish from the Madeira and Selvagens Archipelagos by Neuro-2a CBA and LC-HRMS. Mar. Drugs 20 (4), 236. https://doi.org/10.3390/md20040236.

Tsumuraya, T., Fujii, I., Inoue, M., Tatami, A., Miyazaki, K., Hirama, M., 2006. Production of monoclonal antibodies for sandwich immunoassay detection of ciguatoxin 51-hydroxyCTX3C. Toxicon: Off. J. Int. Soc. Toxinol. 48 (3), 287–294. https://doi.org/10.1016/j.toxicon.2006.05.014.

Tsumuraya, T., Takeuchi, K., Yamashita, S., Fujii, I., Hirama, M., 2012. Development of a monoclonal antibody against the left wing of ciguatoxin CTX1B: thiol strategy and detection using a sandwich ELISA. Toxicon: Off. J. Int. Soc. Toxinol. 60 (3), 348–357. https://doi.org/10.1016/j.toxicon.2012.04.347.

Tsumuraya, T., Hirama, M., 2025. Anti-ciguatoxin monoclonal antibodies: hapten design, production, ELISA, and treatment of ciguatera poisoning. Chem. Lett. 54. https://doi.org/10.1093/chemle/upaf058.

Tsumuraya, T., Sato, T., Hirama, M., Fujii, I., 2018. Highly sensitive and practical fluorescent sandwich ELISA for ciguatoxins. Anal. Chem. 90 (12), 7318–7324. https://doi.org/10.1021/acs.analchem.8b00519. Jun 19Epub 2018 May 29. PMID: 29770692.

USFDA, 2011. Chapter 6: natural toxins. U.S. Food Drug Adm. Fish Fish. Prod. Hazards Controls Guid. fourth edition http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/Seafood/ucm2018426.htm.

Vernoux, J.P., Lahlou, N., Abbad el Andaloussi, S., Riyeche, N., Magras, L.P., 1985. A study of the distribution of ciguatoxin in individual Caribbean fish. Acta Trop 42, 225–233.

Vernoux, J.P., Lewis, R.J., 1997. Isolation and characterisation of Caribbean ciguatoxins from the horse-eye jack (Caranx latus). Toxicon 35 (6), 889–900.

Wenzl, T., Haedrich, J., Schaechtele, A., Robouch, P., Stroka, J., 2016. Guidance Document on the Estimation of LOD and LOQ for Measurements in the Field of Contaminants in Feed and Food; EUR 28099. European Union, Luxembourg. ISBN 978-992-979-61768-61763.

Wu, J.J., Mak, Y.L., Murphy, M.B., Lam, J.C., Chan, W.H., Wang, M., Chan, L.L., Lam, P. K., 2011. Validation of an accelerated solvent extraction liquid chromatography–tandem mass spectrometry method for Pacific ciguatoxin-1 in fish flesh and comparison with the mouse neuroblastoma assay. Anal, Bioanal.Chem. 400 (9), 3165–3175.

Yasumoto, T., Igarashi, T., Legrand, A.M., Cruchet, P., Chinain, M., Fujita, T., et al., 2000. Structural elucidation of ciguatoxin congeners by fast-atom bombardment tandem mass spectroscopy. J. Am, Chem, Soc. 122, 4988–4989. https://doi.org/10.1021/ja9944204.

Yogi, K., Oshiro, N., Inafuku, Y., Hirama, M., Yasumoto, T., 2011. Detailed LC-MS/MS analysis of ciguatoxins revealing distinct regional and species characteristics in fish and causative Alga from the Pacific. Anal. Chem. 83, 8886–8891.