A smartphone-controlled amperometric immunosensor for the detection of Pacific ciguatoxins in fish

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Abstract

Ciguatoxins (CTXs) are marine neurotoxins produced by microalgae of the genera *Gambierdiscus* and *Fukuyoa*. CTXs may reach humans through food webs and cause ciguatera fish poisoning (CFP). An immunosensor for the detection of Pacific CTXs in fish was developed using multiwalled carbon nanotube (MWCNT)-modified carbon electrodes and a smartphone-controlled potentiostat. The biosensor attained a limit of detection (LOD) and a limit of quantification (LOQ) of 6 and 27 pg/mL of CTX1B, respectively, which were 0.001 and 0.005 µg/kg in fish flesh. In the analysis of fish samples from Japan and Fiji, excellent correlations were found with sandwich enzyme-linked immunosorbent assays (ELISAs), a cell-based assay (CBA) and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Stability of at least 3 months at −20 °C was predicted. In just over 2 h, the biosensor provides reliable, accurate and precise Pacific CTX contents in fish extracts, being suitable for monitoring and research programs.

Keywords: Pacific ciguatoxins (CTXs), fish, biosensor, enzyme-linked immunosorbent assay (ELISA), cell-based assay (CBA), liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

1. INTRODUCTION

Ciguatoxins (CTXs) are potent neurotoxins produced by microalgae of the genera *Gambierdiscus* and *Fukuyoa* (Litaker et al., 2017). These benthic dinoflagellates are grazed by herbivorous fishes, which are then eaten by carnivorous fishes, and therefore may reach human consumers and cause ciguatera fish poisoning (CFP) (Soliño & Costa, 2020). Throughout the transfer of CTXs from the microalgae, metabolization processes in the fish result in CTX transformations increasing the spectrum of CTX derivatives (Ikehara, Kuniyoshi, Oshiro, & Yasumoto, 2017). P-CTX-1 (CTX1B) is the most potent CTX congener, thought to be responsible for the majority of symptoms associated with CFP in the Pacific. Moreover, CTX3C and CTX4A derivatives have been described from the Pacific Ocean (Satake, Murata, & Yasumoto, 1993; Satake, Ishibashi, Legrand, & Yasumoto, 1996). In the Caribbean Sea and the East Atlantic Ocean, structurally different Caribbean CTXs (C-CTXs) are present (Vernoux and Lewis, 1997; Estevez, Leao, Yasumoto, Dickey, & Gago-Martinez, 2019). Indian CTXs (I-CTXs) have been reported from the Indian Ocean, although their structures are unclear (Hamilton, Hurbungs, Jones, & Lewis, 2002; Diogène et al.,

2017). At cellular level, CTXs act on the voltage-gated sodium channels (VGSCs), blocking them in an open position.

CFP is the most common and one of the most relevant seafood-borne diseases worldwide, affecting 10000 to 500000 people per year, and probably more due to under-diagnosis and under-reporting (Friedman et al., 2017; Chinain, Gatti, Darius, Quod, & Tester, 2021). CFP is characterized by severe neurological, gastrointestinal and cardiovascular disorders that usually abate within a few days or weeks but that can persist for months or years (Lehane & Lewis, 2000; Anadon, Ares, Martinez, Martinez-Larranaga, & Martinez, 2021). CTXs are tasteless, colorless, odorless and stable to acid, heat and freezing. Therefore, CFP cannot be prevented by any storage, preparation or cooking methods.

Although *Gambierdiscus* and *Fukuyoa* are endemic from tropical and subtropical regions, they have been recently detected in more temperate regions (Dickey & Plakas, 2010; Tudó et al., 2020b). CFP has geographically expanded, certainly due to a broader distribution of CTX-producing microalgae in temperate waters, resulting in the presence of CTXs in fish from areas where no CFP cases had been reported (e.g., Madeira and the Canary Islands). This microalgae expansion is potentially due to aquatic environmental changes such as sea surface temperature increase, but also other causes such as transport via ship's ballast water. In addition, more CFP cases have been recorded in countries from temperate areas due to international seafood trade and travel to endemic areas (Lange, Snyder, & Fudala, 1992).

In the Pacific, mild CFP outbreaks occurred after exposure to fish containing 0.1 µg/kg P-CTX-1 equivalent toxicity estimated using the mouse bioassay (MBA) (Lehane and Lewis, 2000). In the Caribbean, a value of 1.0 µg/kg C-CTX-1 equivalent toxicity was estimated also using the MBA (Vernoux and Lewis, 1997). Applying a 10-fold safety factor, the United States Food and Drug Administration has proposed guidance levels of 0.01 µg/kg of P-CTX-1 (CTX1B) equivalents and 0.1 µg/kg of C-CTX-1 equivalents in fish (US FDA, 2020). This value has also been recommended by the European Food Safety (EFSA) to cover all CTX-group toxins that could be present in fish (EFSA, 2010). In Europe, the current legislation only requires that no fish products with CTXs are placed on the market (Commission Regulation (EC) No. 853/2004). Australia and New Zealand provide guidelines on possible ciguateric fish species and areas (FSANZ, 2006). In Japan, some fish species associated to CFP are banned to import and the local governments recommend rejecting certain fish species caught in Japan (MHW, 2001; Oshiro et al., 2021a).

Detection of CTXs is a big challenge because of the complexity and variety of their chemical structures, the long and tedious protocols for their extraction from natural samples, and the extremely sensitive analysis required to detect less than 0.01 µg/kg (Loeffler et al., 2021). Among the different detection methods, the MBA had been the most used (Hoffman, Granade, & McMillan, 1983), followed by the cell-based assay (CBA) (Manger, Leja, Lee, Hungerford, & Wekell, 1993) and instrumental analysis techniques such as liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) (Lewis, Yang, & Jones, 2009; Yogi, Oshiro, Inafuku, Hirama, & Yasumoto, 2011). Some receptor-binding assays (RBAs) for CTXs have also been approached (Dechraoui et al., 2005). Polyclonal (Hokama, Banner, & Boylan, 1977) and monoclonal (Hokama, Hong, Isobe, Ichikawa, & Yasumoto, 1992) antibodies have been produced for the development of immunoassays for the detection of CTXs. These immunoassays were later formatted into the immunostrip tests with the commercial names Cigua-Check and Ciguatect. However, their reliability was dubious (Dickey, Granade, & McClure, 1994). Since 2006, Tsumuraya and co-workers have successfully produced new monoclonal antibodies (mAbs) against rationally designed synthetic CTX haptens and have developed sandwich

enzyme-linked immunosorbent assays (ELISAs) for some Pacific CTXs (CTX1B, 54-deoxyCTX1B, CTX3C and 51-hydroxyCTX3C) (Tsumuraya et al., 2006; Tsumuraya, Fujii, & Hirama, 2010; Tsumuraya, Takeuchi, Yamashita, Fujii, & Hirama, 2012; Tsumuraya, Sato, Hirama, & Fujii, 2018). These mAbs, which are highly specific and sensitive, circumvent the cross-reactivity problems previously observed with other antibodies (Hokama, Banner, & Boylan, 1977).

The different techniques for the detection of CTXs have advantages and limitations. The MBA provides toxicological information of a sample, is simple to perform and does not require extensive sample conditioning, but is not specific and sensitive enough, requires high amounts of fish tissue, and has ethical concerns. The CBA also provides a composite toxicological response, since it is based on the mode of action of CTXs on cells (which interact at site 5 of the VGSCs, leaving them in a permanent open state), and is highly sensitive, therefore requiring small amounts of fish tissue and CTX calibrants, but may not be specific (it also recognizes brevetoxins and other potentially interfering compounds with the same mode of action), is time-consuming, has high variability, and requires standardization, which is difficult to achieve when using living material. Both toxicological approaches respond to many CTX congeners, providing a composite toxicity evaluation. Instrumental analysis techniques, where the detection is based on physico-chemical and structural properties of CTXs, allow unambiguous quantifications of some CTX congeners, are very specific, and sensitivity may be appropriate, but usually require high amounts of fish tissue, long and tedious sample clean-up processes, are complex, expensive, and highly trained personnel is required. Instrumental analysis provides information on individual compounds, but it may not cover all CTX congeners, due to unavailability of some reference materials. RBAs are appropriate screening tools, also based on the interaction of CTXs with the VGSCs at site 5, and therefore on toxicity, but may be not specific and sensitive enough, and sometimes require radioactivity detection. Immunoassays are sensitive and easy to implement, but require high-quality antibody production, may be affected by cross-reactivity with other compounds, and provide a composite response related to the structure. Combination of techniques is often the key to success.

The threat that the presence of CTXs in fish poses to human health and some limitations of the current analytical methodologies highlight the need for fast and reliable screening methods. To this purpose, our group has recently developed an electrochemical immunosensor and has applied it to the analysis of fish (Leonardo et al., 2020) and microalgae samples (Gaiani et al., 2020; Tudó et al., 2020a). This immunosensor uses the highly specific and sensitive mAbs produced by Tsumuraya and co-workers, previously mentioned. The work presented herein aims to simplify even more the protocol and make a portable biosensor. To this purpose, capture mAbs were immobilized on multiwalled carbon nanotube (MWCNT)-modified carbon electrodes instead of magnetic beads. The sandwich assay was then conducted, and amperometric signals were measured with a smartphone-controlled potentiostat (Fig. 1). The electrochemical immunosensor was applied to the analysis of fish samples from Japan and Fiji, and results were compared with those obtained with sandwich ELISAs, a CBA and LC-MS/MS.

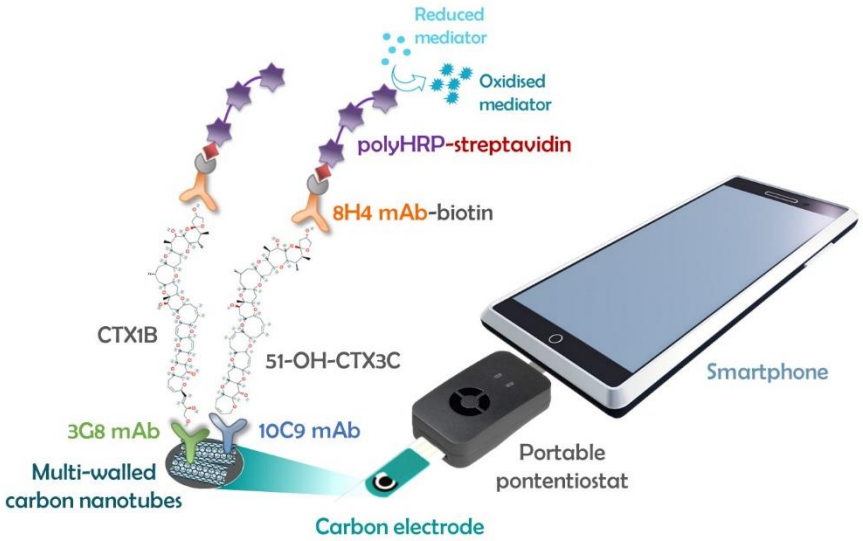

Figure 1. Representation of the smartphone-controlled biosensor for the detection of Pacific CTXs.

2. MATERIALS AND METHODS

2.1. Reagents and materials

For the extraction of CTXs from fish flesh, acetone, hexane, and ethyl acetate of the Primepure grade, diethyl ether of guaranteed reagent grade, and methanol (MeOH) and acetonitrile (ACN) of liquid chromatography-mass spectrometry (LC-MS) grade were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). For the biosensor, 4-morpholineethanesulfonic acid (MES) hydrate, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), potassium phosphate monobasic, potassium phosphate dibasic, potassium chloride, Tween®-20, bovine serum album (BSA), anti-mouse IgG (whole molecule)-horseradish peroxidase antibody produced in rabbit (IgG-HRP), and 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate were purchased from Sigma-Aldrich (Tres Cantos, Spain). PolyHRP-streptavidin was obtained from Thermo Fisher (Barcelona, Spain). Milli-Q® water (Millipore, Bedford, MA, USA) was used to prepare all solutions. For the ELISAs, F96 Nunc MaxiSorp immunoplate microtiter wells were obtained from Thermo Fischer Scientific (Waltham, MA, USA). Potassium phosphate monobasic, sodium phosphate dibasic dodecahydrate, sodium chloride, sucrose, Tween®-20, tris(hydroxymethyl)aminomethane, hydrochloric acid and dimethyl sulfoxide (DMSO) of guaranteed reagent grade were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Proclin 300 was obtained from Sigma-Aldrich (St. Louis, MO, USA). Blocking Reagents N101 and N102 were purchased from NOF Corporation (Tokyo, Japan). The buffer solutions were prepared as in previous works (Tsumuraya, Sato, Hirama, & Fujii, 2018). *p*-Nitrophenyl phosphate (Alkaline Phosphatase Yellow Liquid Substrate System for ELISA) was obtained from Sigma (Kanagawa, Japan) and AttoPhos AP Fluorescent Substrate System was obtained from Promega (Madison, WI, USA). For the CBA, neuroblastoma murine cells (Neuro-2a) were purchased from ATCC LGC standards (USA). Fetal bovine serum (FBS), L-glutamine solution, ouabain, veratridine, phosphate buffered saline (PBS), penicillin, streptomycin, RPMI-1640 medium, sodium pyruvate and thiazolyl blue tetrazolium bromide (MTT) were purchased from Merck KGaA (Gernsheim, Germany). DMSO was purchased from Chemlab (Spain). For LC-MS/MS analysis, ammonium formate solution (1 M) and formic acid

were of high-performance liquid chromatography (HPLC) grade (Wako Chemical Industry, Ltd., Osaka, Japan), and Milli-Q® water (Millipore, Bedford, MA, USA) was used.

Stock 3G8, 10C9 and 8H4 mAb solutions, previously prepared at Osaka Prefecture University (OPU), were at 6.88, 6.61 and 4.24 mg/mL, respectively. Biotin labelling of the 8H4 mAb (for the biosensor) was performed with the EZ-Link™ NHS-PEG4 Biotinylation Kit from Thermo Fisher (Barcelona, Spain). Alkaline phosphatase (ALP) labelling of the 8H4 mAb (for the ELISAs) was performed with the LYNX Rapid Alkaline Phosphatase Antibody Conjugation Kit (BIO-RAD, California, USA).

The purified CTX1B used for the biosensor and the CBA calibration curves was obtained from Prof. Richard J. Lewis (The Queensland University, Australia) and cross-calibrated in relation to the NMR-quantified CTX1B reference material provided by Prof. Takeshi Yasumoto (Japan Food Research Laboratories (JFRL)). The CTX1B and CTX3C calibrants used for the ELISA were prepared from synthesized ones at Tohoku University (Hirama et al., 2001; Inoue et al., 2006) and cross-calibrated in relation to CTX calibrants provided by JFRL. The mixed-CTXs calibrant solution used in LC-MS/MS analysis, consisting of CTX1B, 52-*epi*-54-deoxyCTX1B, 54-deoxyCTX1B, CTX4A, CTX4B, 2,3-dihydroxyCTX3C, 51-hydroxyCTX3C, 49-*epi*CTX3C and CTX3C, was prepared at the National Institute of Health Sciences (NIHS) using purified or semi-purified CTXs provided by Prof. Takeshi Yasumoto (JFRL). The levels of CTXs in the mixture were determined using the CTX1B (43.3 ± 1.3 ng), 52-*epi*-54-deoxyCTX1B (58.4 ± 2.5 ng), CTX4A (55.1 ± 5.2 ng), 51-hydroxyCTX3C (45.3 ± 7.2 ng) and CTX3C (38.5 ± 2.6 ng) calibrants provided by JFRL. Since no NMR-quantified reference materials were available for the analogues of 54-deoxyCTX1B, CTX4B, 2,3-dihydroxyCTX3C and 49-*epi*CTX3C, they were quantified using the calibration curves of 52-*epi*-54-deoxyCTX1B, CTX4A, 51-hydroxyCTX3C and CTX3C, respectively.

Screen-printed carbon electrodes modified with carboxyl-functionalized multi-walled carbon nanotubes (DRP-110CNT) were purchased from Metrohm DropSens S.L. (Oviedo, Spain). Amperometric measurements were performed with a PalmSens Sensit Smart potentiostat (Houte, The Netherlands). PalmSens PStouch software was used to collect and evaluate data.

2.2. Fish samples

Fish samples used were judged as both non-contaminated and CTXs-contaminated by preliminary analysis by LC-MS/MS or our previous studies (Oshiro et al., 2021a; Oshiro, Tomikawa, Kuniyoshi, Ishikawa, & Toyofuku, 2021b; Oshiro et al., 2021c). Non-contaminated specimens (specimens 1-4) included *Lutjanus bohar* (two two-spot red snappers from Okinawa), *Lutjanus monostigma* (one one-spot snapper individual from Kagoshima) and *Variola louti* (one yellow-edged lyretail from Ehime) from Japan. Contaminated specimens (specimens 6-17) included *L. bohar* (five two-spot red snappers from Okinawa and two from Wakayama), *L. monostigma* (one one-spot snapper from Kagoshima and two from Okinawa), *V. louti* (one yellow-edged lyretail from Okinawa) and *Variola albimarginata* (one white-edged lyretail from Okinawa) from Japan. Furthermore, one individual of *Gymnothorax javanicus* (giant moray) (specimen 5) purchased at the Viti Levu Island, Fiji (Oshiro, Tomikawa, Kuniyoshi, Ishikawa, & Toyofuku, 2021b) was used. In Figure S1, a map with the fish collection sites is shown, and in Table S1, the weight and length of the fishes are provided. Those fishes were destined for human consumption and obtained from fishers; some of them had been disapproved and recalled from the market (Table S1). LC-MS/MS analysis of this specimen had revealed CTXs in the ACN eluate solution, but not in the MeOH eluate solution (Oshiro, Tomikawa, Kuniyoshi, Ishikawa, & Toyofuku, 2021b). In the current work, this MeOH eluate solution was used to evaluate the

CTX1B recovery, as no other *G. javanicus* specimens were available. All specimens used were raw (uncooked) flesh except for specimen 12 which was stewed with soy source ("Nitsuke" in Japanese).

2.3. CTXs extraction

The fish extracts were prepared at NIHS as described in previous studies (Oshiro et al., 2021a; Oshiro, Tomikawa, Kuniyoshi, Ishikawa, & Toyofuku, 2021b; Oshiro et al., 2021c). The fish flesh (skin not included) (5 g) was extracted with acetone (15 mL, twice), and the combined extracts were evaporated to remove acetone. The remaining aqueous portions were partitioned with diethyl ether (5 mL, twice), and the organic layer was collected and dried completely. The dried materials were dissolved in 90% MeOH (*v/v*, 1.5 mL), defatted with hexane (3 mL, twice) and the remaining solution was dried completely to obtain the crude extract. The crude extract was dissolved in ethyl acetate-MeOH (9:1 *v/v*, 5 mL) and passed through a Florisil cartridge column (500 mg, GL Sciences Inc., Tokyo, Japan). The eluate solution was dried, and the residue was dissolved in ACN (5 mL) and applied to a primary and secondary amine (PSA) cartridge column (200 mg, GL Sciences Inc., Tokyo, Japan) and MeOH (3 mL) was applied to the column. Both ACN and MeOH eluate solutions were dried and dissolved in MeOH (1 mL), which contained 5 g equivalents of fish flesh, and analyzed with LC-MS/MS. Three 250-µL portions of the MeOH eluate solutions were taken into screw cap vials (in the cases of samples 12, 16 and 17, the volumes taken were 200, 100, and 150 µL, respectively), and dried under a nitrogen stream. The ACN eluate solutions of specimens 7 and 8 were analyzed, since some CTX3C analogues were detected in our previous study by LC-MS/MS.

One of three sets of the dried fish extract samples was shipped to OPU with dry ice to analyze CTXs by the ELISAs. Another set was shipped to IRTA to analyze CTXs by the electrochemical biosensor and the CBA. The remaining set of the samples were analyzed by LC-MS/MS at NIHS. These fish extracts were dissolved with MeOH, ACN or DMSO to make fish extract solutions at 5 g equivalents of fish flesh/mL.

2.4. Electrochemical immunosensor

The protocol for the construction of the electrochemical immunosensor and the analysis of samples is depicted in Figure 2. The carboxyl groups of the electrodes were activated by incubation with 25 µL of 50 mg/mL EDC and 25 µL of 50 mg/mL NHS (both in 25 mM MES, pH 5.0) for 30 min. After washing, 50 µL of a 3G8+10C9 mAb mixture in MES (from 1/50 to 1/2000 dilution for protocol optimization and 1/500 dilution (14 and 13 µg/mL for 3G8 and 10C9, respectively) for the final biosensor) was incubated for 1 hour. Electrodes were washed and exposed to 50 µL of purified CTX1B (from 800 to 3.12 pg/mL for the calibration curves) or fish extract (at 5, 2.5 and 1.25 g equivalents of fish flesh/mL) in 100 mM PBS, pH 7.2, containing 0.05% *v/v* Tween®-20, both previously evaporated, for 30 min. After washing, a blocking step was performed with 50 µL of PBS-Tween-BSA (PBS-Tween containing 2% *w/v* BSA) for 30 min. Electrodes were washed and incubated with 50 µL of biotin-8H4 mAb in PBS-Tween-BSA (from 1/100 to 1/5000 dilution for protocol optimization and 1/1000 dilution for the final biosensor) for 30 min. After washing, electrodes were incubated with 50 µL of polyHRP-streptavidin in PBS-Tween-BSA (from 1/100 to 1/1000 dilution for protocol optimization and 1/1000 dilution (2 µg/mL) for the final biosensor) for 30 min. Finally, electrodes were washed and 50 µL of TMB was added. After a 2-min incubation, the TMB reduction current was measured using amperometry, applying −0.2 V (*vs.* Ag) for 5 s. The negative reaction current intensities were

taken and expressed in absolute value. All incubations were performed at room temperature. Measurements were performed in triplicate.

2.5. ELISAs

Three different sandwich ELISAs were performed: a colorimetric ELISA for the CTX1B series, a fluorescent ELISA for the CTX1B series, and a fluorescent ELISA for the CTX3C series (Tsumuraya, Sato, Hirama, & Fujii, 2018). Briefly, to detect the CTX1B series, microtiter wells were coated with 100 µL of 3G8 mAb (10 µg/mL) in PBS overnight. Then, 400 µL of blocking buffer was incubated for 1 h. After washing, 100 µL of CTX1B calibrant serially diluted with D1 buffer or fish extract (redissolved in DMSO at 5 g equivalents of fish flesh/mL and 10-fold diluted with D1 buffer) was added and incubated for 1 h. After washing, 100 µL of ALP-8H4 (2 µg/mL) in D2 buffer was incubated for 1 h. After washing, 100 µL of *p*-nitrophenyl phosphate were incubated for 10-30 min and then, absorbance was measured at 405 nm. For the samples with low CTX1B contents, the fluorescent ELISA was applied. To detect the CTX3C series, the 10C9 mAb was used to coat the microtiter wells, and detection was performed using the fluorescent ELISA. All incubations were performed at room temperature. Measurements were performed in triplicate.

2.6. CBA

The CBA was performed as previously described (Diogène et al., 2017). Briefly, Neuro-2a cells (ATCC, CCL131) were seeded in a 96-well microplate in 200 µL of RPMI medium containing 5% *v/v* fetal bovine serum (RPMI-FBS) at 34000 cell/well and incubated at 37 °C in a 5% CO_2 humid atmosphere for 24 h. Prior to exposure to purified CTX1B (from 120 to 0.96 pg/mL for the calibration curves) or fish extract (at 5, 2.5 and 1.25 g equivalents of fish flesh/mL), some Neuro-2a cells were pre-treated with 100 µM ouabain and 10 µM veratridine (concentration in the wells). Purified CTX1B or fish extract were dried, reconstituted in 200 µL of RPMI-FBS medium, serially diluted, and 10 µL was added to the wells with and without ouabain/veratridine pre-treatment (final volume of 230 µL). After 24 h, cell viability was measured using the MTT assay (Manger, Leja, Lee, Hungerford, & Wekell, 1993) recording the absorbance at 570 nm with a Microplate Reader KC4 from BIO-TEK Instruments Inc. (Winooski, VT, USA). Measurements were performed in triplicate.

2.7. LC-MS/MS analysis

LC-MS/MS analyses were caried out as described previously reported (Oshiro et al. 2021a; Oshiro, Tomikawa, Kuniyoshi, Ishikawa, & Toyofuku, 2021b) using an Agilent (Santa Clara, CA) 1290 HPLC system coupled to an Agilent 6460 Triple Quadrupole MS instrument. Dried samples were dissolved in respective volume of MeOH (250, 200, 150 or 100 µL as described in above) to make 5 g equivalents of fish flesh/mL solutions. A volume of 5 µL of solution was injected into a Zorbax Eclipse Plus C18 column (2.1 × 50 mm id, 1.8 µm, Agilent Technologies, Santa Clara, CA, USA), at 40 °C. The eluate solution A was water containing 5 mM ammonium formate and 0.1% formic acid and the eluate solution B was MeOH. The gradient system (gradient I) was as follows: 0.0-0.25 min (60%B), 0.25-0.50 min (60-75%B), 0.50-12.0 min (75%B), 12.0-14.0 min (90%B), 14.1-20 min (100%B). When the presence of an interfering substance was suspected, the sample was re-analyzed using another gradient system (gradient II: 0-0.25 min (50%B), 0.25-0.5 min (50-65%B), 0.5-25 min(65-80%B), 25-27 min (80%B), 27-33 min (100%B)). The flow rate was 0.4 mL/min. The target toxins were ionized with electron spray ionization (ESI) equipped with Agilent Jet Stream, and positive ions were monitored with a multiple reaction monitoring (MRM) mode. Since $[M+Na]^+$ ions were stable and gave no fragment ions, $[M+Na]^+$ of each analogue

was set for both precursor and product ions ($[M+Na]^+ > [M+Na]^+$), with high collision energy to achieve sensitive analysis. Optimized MS parameters were: dry gas N_2, 300 °C, 10 L/min; nebulizer gas N_2, 50 psi; sheath gas N_2, 380 °C, 11 L/min; capillary voltage 5000 V; fragmentor voltage 300 V; collision gas N_2, collision energy 40 eV. The limit of detection (LOD) and the limit of quantitation (LOQ) values of CTX1B, 52-*epi*-54-deoxyCTX1B, and 54-deoxyCTX1B were 0.001 µg/kg and 0.005 µg/kg, respectively. Since no reference material of 2,3,51-trihydroxyCTX3C was available, it was deduced from the *m/z* of $[M+Na]^+$ and retention time, as described in previous manuscripts (Yogi, Oshiro, Inafuku, Hirama, & Yasumoto, 2011; Oshiro et al., 2021c), and quantified using a calibration curve of the 51-hydroxyCTX3C.

3. RESULTS AND DISCUSSION

3.1. Immunosensor optimization

The concentration of the different components of the biosensor was optimized. First, the capture mAb mixture (3G8+10C9) dilution was optimized using non-limiting detector mAb (8H4) and polyHRP-streptavidin dilutions and 400 pg/mL of CTX1B. Several dilutions were tested (from 1/50 to 1/2000). Electrochemical signals were constant from 1/50 to 1/500 and started to decrease at 1/1000 and more drastically at 1/2000. Therefore, the 1/500 3G8+10C9 dilution was chosen for subsequent experiments. Then, the detector mAb (biotinylated 8H4) dilution was tested (from 1/100 to 1/5000). No differences in the electrochemical signals were observed from 1/100 to 1/2000, which started to decrease at 1/5000. Additionally, non-specific adsorption (response in the absence of CTX1B) was observed at 1/100 and 1/200 but disappeared at lower mAb concentrations. Therefore, the 1/1000 biotinylated 8H4 dilution was chosen for subsequent experiments (to ensure that the biotinylated 8H4 mAb concentration was not a limiting parameter). Finally, several polyHRP-streptavidin dilutions were tested (from 1/100 to 1/2000). In this case, electrochemical signals decreased with the dilution and no plateau was observed. Non-specific adsorption was observed from 1/100 to 1/500 but disappeared at lower polyHRP-streptavidin concentrations. The best signal-to-noise ratio was observed at 1/1000 polyHRP-streptavidin dilution, which was chosen for further experiments.

Once the experimental parameters were optimized, the calibration curve for CTX1B was constructed (Fig. 3). CTX1B was chosen as a model CTX because the only existing guidance level, provided by the FDA, is expressed in CTX1B equivalents (≤0.01 µg/kg). No saturation was observed at high CTX1B concentrations, indicating that the working range of the immunosensor is probably wider than the tested concentrations. Higher CTX1B concentrations were not used because of the high price and scarcity of CTX1B. Nevertheless, the working range was well over 2 orders of magnitude. LOD and LOQ values of 6 and 27 pg/mL, respectively, were obtained. These values are similar to those obtained with the magnetic bead-based immunosensor (2 and 3 pg/mL) (Leonardo et al., 2020). A considerable advantage of the current biosensor is that 1/500 dilution is used for the immobilization of the capture mAbs, which is 10-fold lower than in our previous work and represents a substantially lower cost. Previous works suggest that the electrodes modified with capture mAbs could be reused (Leonardo et al., 2018), saving both mAbs and screen-printed carbon electrodes, and further reducing the cost.

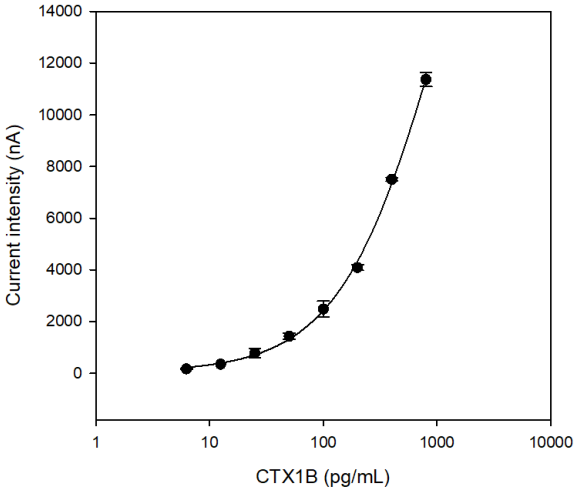

Figure 3. Calibration curve for CTX1B obtained using the smartphone-controlled biosensor (reduction current intensities are in absolute value). Values are background-subtracted. Measurements were performed in triplicate.

3.2. Immunosensor performance in the presence of fish matrix

To evaluate the effect of the fish flesh matrix components on the immunosensor performance, MeOH eluate solutions from PSA cartridge columns of *L. bohar*, *L. monostigma*, *V. louti* and *G. javanicus* (at 5 g equivalents of fish flesh/mL), specimens considered as negative by LC-MS/MS (no CTXs detected), were spiked with 400 pg/mL of CTX1B (equivalent to 0.08 µg/kg in the fish flesh). The purpose of this experiment was to ensure that the biosensor was responding to Pacific CTXs in fish extracts. Spiked extracts were analyzed with the biosensor and results were compared to those obtained in buffer. Recovery values were 99.1±3.4, 104.4±0.7, 96.9±0.2 and 92.3±1.9% for *L. bohar*, *L. monostigma*, *V. louti* and *G. javanicus*, respectively (the small amounts of CTX3C (0.003 µg/kg) detected with the fluorescent ELISA for the CTX3C series in the MeOH eluate solution of this *G. javanicus* specimen, certainly due to the lower LOD (see next section), are not affecting the calculation of the recovery with the biosensor). These values indicate that components of purified extracts of fish flesh matrix are not interfering in the assay. The absence of matrix effects is another advantage over our previous work, where recovery values between 58 and 89% were obtained at different fish flesh matrix concentrations (Leonardo et al., 2020). This lower interference from the fish flesh matrix components in the current work is certainly due to the extract preparation protocol used, which included several purification steps and resulted in cleaner solutions.

Considering that a natural sample is analyzed at 5 g equivalents of fish flesh/mL and that no significant matrix effects are present, effective LOD and LOQ values of 0.001 and 0.005 µg/kg of CTX1B in fish flesh, respectively, are obtained. These values are lower than the FDA guidance level of 0.01 µg/kg. The decision limit (CCα), which is the concentration at which the biosensor can conclude with a statistical certainty of 1 – α (95 %) that the FDA guidance level has been truly exceeded (5% of false positive results), was 0.013 µg/kg. The detection capability (CCβ), which is the concentration at which the biosensor is able to detect concentrations at the FDA guidance level with a statistical certainty of 1 – β (95 %) (5% of false negative results), was 0.014 µg/kg. Therefore, the biosensor is appropriate as both a screening tool and a quantification method for positive samples.

Repeatability and reproducibility of the biosensor were evaluated by performing multiple measurements on the same day (intraday precision) and different days (interday precision), respectively (Gerssen, Van Olst, Mulder, & De Boer, 2010). Relative standard deviation (RSD) values for measurements performed on the same day were 4.9, 3.1 and 4.4% (N=3) at 0.015, 0.010 and 0.005 µg/kg of CTX1B in fish flesh, respectively. RSD values for the measurements performed on different days with different mAb-modified electrodes were 8.7, 7.6 and 18.8% (N=5) at 0.015, 0.010 and 0.005 µg/kg of CTX1B in fish flesh, respectively. These values are appropriate and show the high performance of the whole procedure including both immunosensor preparation and electrochemical measurement.

3.3. Analysis of natural samples

The MeOH eluate solutions from PSA cartridge columns of fish samples from Japan and Fiji were analyzed with the immunosensor and results were provided in CTX1B equivalent contents using the corresponding calibration curve. Results were compared to those obtained with the ELISAs, the CBA and LC-MS/MS (Table 1).

The biosensor and the ELISAs are based on the same recognition principle. However, these two strategies differ in different aspects. Whereas the biosensor uses two capture antibodies and provides a composite response from the recognized Pacific CTXs (CTX1B, 54-deoxyCTX1B, CTX3C and 51-hydroxyCTX3C), these ELISAs are performed with single antibodies and can discriminate between congeners of the CTX1B and CTX3C series. The solid support where capture antibodies are immobilized is also different: screen-printed carbon electrodes modified with MWCNTs in the biosensor and microtiter plates in the ELISAs. Finally, whereas the biosensor uses amperometry as a detection method, the ELISAs use colorimetry or fluorescence. Despite these differences, when comparing the CTX contents between these two methods, the correlation is excellent (r=0.9455; P<0.001) (Fig. 4A). Although the slope (1.3139) indicates that in general the CTXs contents obtained with the biosensor are higher than those provided by the ELISAs, at an individual level these higher contents are only appreciated in the most toxic samples. It is necessary to mention that, as explained in the experimental section, fish samples were split into three vials each, dried and shipped to the different laboratories participating in this work. Therefore, shipment and resuspension could be a source of differences in the CTX contents. Nevertheless, taking all the issues into account, the agreement is good enough.

When comparing the biosensor and the CBA, it is necessary to take into account that they have different detection principles. Whereas the CBA provides a toxicological response due to the effect of CTXs on the VGSCs of Neuro-2a cells, this biosensor responds to the structural interaction between CTXs and the mAbs. Fish specimens 1 to 5, considered as negative by the biosensor, were also negative by the CBA and LC-MS/MS (negative meaning no CTXs detected). As can be observed in Fig. 4B, the correlation between the biosensor and the CBA is excellent (r=0.9879; P<0.001) and the CTXs contents obtained with the biosensor are in general only slightly lower than those provided by the CBA (slope of 0.8957). In this case, the same set of extracts was used for the analysis with the biosensor and the CBA. It is interesting to note that in our previous work (Leonardo et al., 2020), although the correlation was good, the magnetic bead-based immunosensor provided much lower CTX1B equivalent contents than the CBA. In that work, we hypothesized that the CBA could be detecting a higher number of CTXs or even other compounds different from CTXs that also activate VGSCs (this could also happen in this work, although in a lower extent). Additionally, the cross-reactivity factors (CRFs) for the different CTX congeners in the immunosensor could not be necessarily the same as the toxic equivalency factors (TEFs) in the CBA. The different origin of the positive samples, from La

Réunion and Maurice Islands (Indian Ocean) in the previous work and from Japan (Northwest Pacific Ocean) in the current work, and consequently, the probably different CTX analogues profiles may explain this issue. In fact, the mAbs used in this work were produced against synthetic fragments of CTXs from the Pacific Ocean.

Regarding the comparison between the biosensor and LC-MS/MS, although both methods are based on a structural recognition, the biosensor only detects the Pacific CTX congeners recognized by the mAbs (which, in the MeOH eluate solutions used in this work, are CTX1B and 54-deoxyCTX1B). LC-MS/MS analysis revealed the presence of CTX1B, 52-*epi*-54-deoxyCTX1B and/or 54-deoxyCTX1B in fish specimens 6 to 17 (in specimens 9 and 10 some of them at contents below the LOQ, and in specimen 11 only trace amounts of 54-deoxyCTX1B). The biosensor also provided positive results for fish specimens 6 to 17, with contents below the LOQ for specimens 10 and 11. Additionally, LC-MS/MS analysis showed the presence of 2,3,51-trihydroxyCTX3C and 2,3-dihydroxyCTX3C in specimens 7 and 8. In the evaluation of the correlation between these two techniques, the application of CRFs of the individual CTX congeners detected by LC-MS/MS is desired to calculate the sum of CTX1B equivalents. Although CRFs may depend on the concentration of the capture mAbs and the immunosensing strategy format, among other experimental conditions, a CRF of 1 was assumed for 54-deoxyCTX1B, which is the CRF found in the fluorescent sandwich ELISA (Tsumuraya, Sato, Hirama, & Fujii, 2018). However, 52-*epi*-54-deoxyCTX1B is recognized in a much lower extent by the mAbs, and 2,3,51-trihydroxyCTX3C and 2,3-dihydroxyCTX3C are not supposed to be recognized. Therefore, a CRF of 0 was applied for these three congeners identified by LC-MS/MS to compare the quantifications of CTXs between the immunosensor and LC-MS/MS (in samples with high 52-*epi*-54-deoxyCTX1B contents, a CRF for this CTX congener, even if low, may need to be applied). As can be observed in Fig. 4C, the correlation is very good (r=0.8683; P<0.001) and the CTXs contents obtained with the biosensor are in general only slightly lower than those provided by LC-MS/MS analysis (slope of 0.8513). This good correlation suggests that shipment of dried fish extracts and further resuspension did not affect CTXs stability.

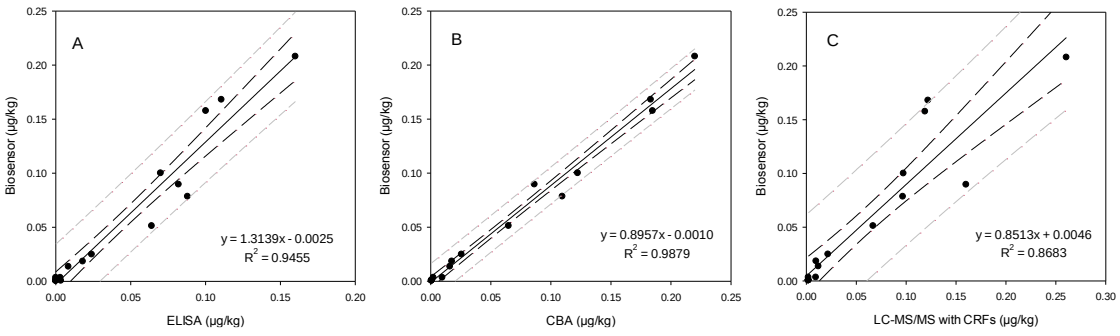

Figure 4. Correlations between CTXs contents (µg/kg) in the MeOH eluate solutions obtained using the ELISAs and the biosensor (A), the CBA and the biosensor (B), and LC-MS/MS and the biosensor (C). Black lines contain the 95% confidence band. Grey lines contain the 95% prediction band.

The ACN eluate solutions of samples 7 and 8 were analyzed by all techniques. The biosensor showed very low CTX1B equivalent contents (0.004 and 0.006 µg/kg, respectively), which were similar to those provided by the fluorescent ELISA for the CTX3C series (0.005 and 0.007 µg/kg, respectively). The CBA also showed low CTX-like toxicity (0.009 and 0.006 µg/kg, respectively), and LC-MS/MS analysis revealed the presence of small amounts of 2-hydroxyCTX3C (0.006 µg/kg

and <LOQ, respectively). Regarding the strategies with the antibodies, the recognition of the left wing of the CTX3C series by the mAb 10C9 is very specific. Therefore, the contents detected with the biosensor and the ELISA are certainly not due to 2-hydroxyCTX3C. One possible explanation is the presence of CTX3C or 51-hydroxyCTX3C at concentrations below the LOD of LC-MS/MS, but detectable with the biosensor and the ELISA.

It is necessary to keep in mind that the biosensor uses a mixture of capture mAbs –the 3G8 mAb, which binds to the left wing of CTX1B and 54-deoxyCTX1B, and the 10C9 mAb, which binds to the left wing of CTX3C and 51-hydroxyCTX3C–, and a detector mAb –the 8H4 mAb, which binds to the right wing of CTX1B, CTX3C, 54-deoxyCTX1B, and 51-hydroxyCTX3C–. Regardless the fact that in the samples analyzed in this work CTX1B-type toxins were more abundant, this dual detection capability can be interesting in the analysis of fish from regions where CTX3C-type toxins co-occur with CTX1B-type toxins or are even more abundant (Oshiro, Tomikawa, Kuniyoshi, Ishikawa, & Toyofuku, 2021b) or in the analysis of *Gambierdiscus* cells, where both types of CTXs can also be present (Longo et al., 2019; Gaiani et al., 2020). The establishment of CRFs and TEFs for all CTX congeners will certainly help to better understand the performance of the biosensor and the CBA. This is a pending task, limited by the availability of standards of some CTX congeners.

3.4. Storage stability

To investigate the possibility of shortening the protocol time, the storage stability of the immobilized detector mAbs at room temperature, 4 and −20 °C for 17 days was evaluated. Electrochemical signals rapidly decreased at room temperature, but they were constant at 4 and −20 °C until the end of the experiment, demonstrating the stability of the functionalized electrodes at these two temperatures. These results were used to predict the shelf life using the Q Rule method (Anderson & Scott, 1991) according to the equation:

$$\text{predicted stability (time)} = \text{real stability (time)} \times (Q10)^{n}$$

where *n* is the temperature change divided by 10, and the value of Q10 is typically set at 2, 3, or 4, which correspond to reasonable activation energies. Taking into account that the functionalized electrodes were stable for at least 17 days at 4 and −20 °C, and considering a *n* value of 2.4 and a conservative Q10 value of 2, the predicted stability at −20 °C is of at least 3 months. These results indicate that a pool of modified electrodes can be prepared on the same day and stored until use, which reduces the assay time approximately from 3,5 to 2 h.

3.5. Applicability

In this work, we have shown that the developed biosensor is a very promising tool to assess the presence of Pacific CTXs in fish. The effective LOD and LOQ values obtained with the smartphone-controlled biosensor are below the FDA guidance level of 0.01 µg equivalents of CTX1B/kg. Therefore, the biosensor can easily discriminate between samples above and below this threshold and be used as both screening and quantification tool. If simple screening is pursued, only one positive (e.g., at the FDA level) and one negative control need to be included in the analysis (instead of the whole calibration curve), reducing time and cost. Although confirmatory analysis of positive samples with instrumental analysis techniques is advised, the high specificity of the antibodies together with the good reproducibility of the quantifications provide reliability to the assessment. It is fair to mention that the antibodies used in this work are highly specific for some Pacific CTXs and are not able to recognize other CTXs (or may recognize some but with much lower cross-reactivity). This fact limits the geographical area of

applicability, but the biosensor is still appropriate for the analysis of samples from regions where the Pacific CTXs recognized by the antibodies are the majority congeners. In Table 2, the performance parameters of the different analytical techniques for detecting Pacific CTXs are compared.

The CTXs extraction and purification protocol, in this work common to all analysis techniques, is the limiting step since it requires time. Nonetheless, due to the storage stability of the biosensor, the detection step can be completed in just 2 h, provided that the fish extracts are ready. In this work, highly purified fish extracts were used, which certainly contributed to the good performance of the techniques. Taking into account that matrix effects in the biosensor and the ELISAs are surely different from those in the CBA or LC-MS/MS, there may be room for simplification of the extraction protocol and consequent reduction in the overall analysis time. This would further speed up the screening process. Therefore, research efforts should focus on the optimization of CTXs extraction and purification protocols combined with the requirements of each analysis technique.

The amount of CTXs calibrants and fish tissue and is also a key issue in the analysis of potentially ciguateric fish as well as other matrixes. This will certainly depend on the sensitivity of the analysis technique and the sample conditioning requirements. The biosensor requires only one CTX standard (CTX1B) and at low concentrations and, in principle, small amounts of fish tissues are needed. However, as previously stated, highly purified samples prepared following LC-MS/MS requirements (and therefore starting from high amounts of fish tissues) have been used in this work. Again, the validation of the biosensor with less purified samples would certainly help to assess their applicability in cases where the amount of sample is limited.

The compact design of the tool, with a miniaturized potentiostat connected to a smartphone, facilitates their use. Staff with basic biochemical/biotechnological skills can easily be trained for biosensor operation. The biosensor can provide *in situ* results thanks to the portable design. Since portable microtiter plate readers are commercially available, the ELISA can also provide *in situ* results. However, as mentioned above, CTXs extraction and purification still need to be optimized to exploit the biosensor and ELISA portability and enable analysis in the field.

The applicability of this biosensor can be expanded to other tissues, such as liver and gonads, after proper validation, to better understand the presence of CTXs in fish where no trace amounts have been found in flesh. Analysis of meal remnants and human body fluids with the biosensor would also be highly interesting. Hence, this tool can help to better assess the risk of ciguatera and improve prevention strategies.

4. CONCLUSIONS

An immunosensor for the detection of Pacific CTXs in fish samples, controlled by a smartphone, was developed. The biosensor, with capture mAbs immobilized on MWCNT-modified carbon electrodes, attained LOD and LOQ values that allow screening and quantification of Pacific CTXs in fish at the FDA guidance level. The CTX1B equivalent contents in fish extracts provided by the biosensor correlated and agreed very well with those obtained with ELISA, CBA and LC-MS/MS, demonstrating the great suitability of this bioanalytical tool for the screening and quantification of CTXs in fish from the Pacific Ocean. Production of antibodies capable of recognizing Caribbean and Indian CTXs is highly desirable to increase the spectrum of detected CTX derivatives and therefore the geographical area of applicability of the biosensor. Additionally, the biosensor requires neither working with living material, which involves maintenance, nor sophisticated or

expensive instrumentation. Thanks to the recording of the electrochemical signal with a smartphone-controlled potentiostat, the biosensor has the distinguishing advantages of being compact, portable and easy to operate. In just a little over 2 h, Pacific CTX contents in fish extracts can be quantified. However, CTXs extraction and purification, long and tedious steps common to all analysis techniques, still need to be optimized to exploit the biosensor portability and enable analysis in the field. Nevertheless, the outstanding performance makes this biosensor suitable as an analytical tool in monitoring and research programs, where accurate and precise CTX quantifications are pursued. The excellent correlation with the CBA guarantees the biosensor reliability in safeguarding human health.

Figure 2. Protocol for the construction of the electrochemical immunosensor and the analysis of samples.

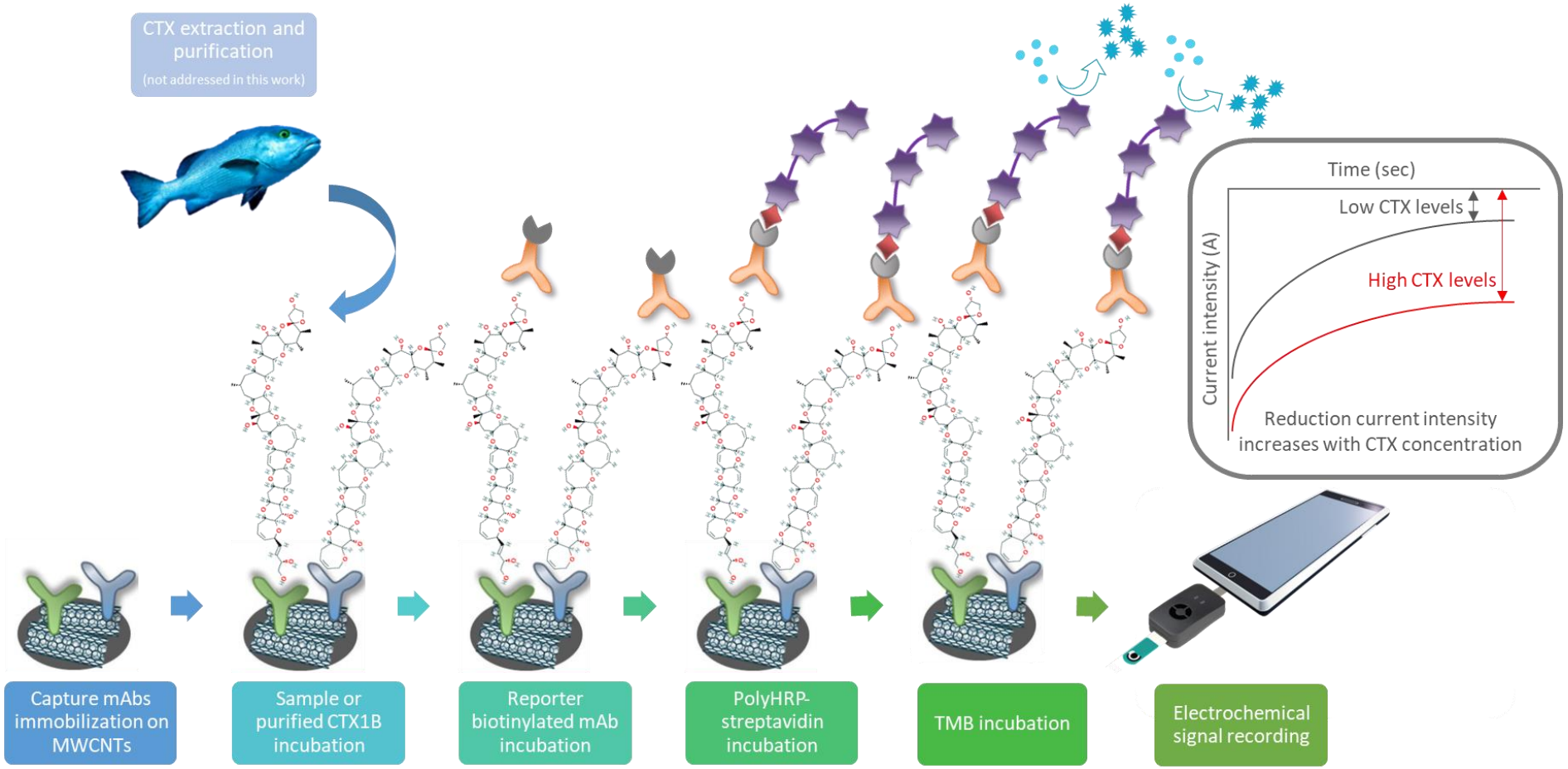

Table 1. CTXs contents (μg/kg) in the MeOH eluate solutions obtained using the smartphone-controlled biosensor, the ELISAs, the CBA and LC-MS/MS.

	Code	Species	Origin	Biosensor	Colorimetric ELISA (CTX1B)	Fluorescent ELISA (CTX1B)	Fluorescent ELISA (CTX3C)	CBA	LC-MS/MS					
									CTX1B	52-*epi*-54-deoxyCTX1B	54-deoxyCTX1B	2,3,51-trihydroxyCTX3C	2,3-dihydroxyCTX3C	Σ all (with CRFs)
1	NIHS-FE20001	*L. bohar*	Okinawa	-		-	-	-	-	-	-	-	-	-
2	NIHS-FE20002	*L. bohar*	Okinawa	-		-	-	-	-	-	-	-	-	-
3	NIHS-FE20003	*L. monostigma*	Kagoshima	-		-	-	-	-	-	-	-	-	-
4	NIHS-FE20004	*V. louti*	Ehime	-		-	-	-	-	-	-	-	-	-
5	NIHS-FE20113	*G. javanicus*	Fiji	-	-	-	0.003	-	-	-	-	-	-	-
6	NIHS-FE20101	*L. bohar*	Okinawa	0.051	0.064		-	0.065	0.029	0.028	0.038	-	-	0.067
7	NIHS-FE20102	*L. bohar*	Wakayama	0.168	0.106		0.005	0.183	0.103	0.012	0.019	0.008	0.009	0.122
8	NIHS-FE20103	*L. bohar*	Wakayama	0.158	0.096		0.004	0.185	0.099	0.010	0.020	0.008	0.007	0.119
9	NIHS-FE20104	*L. bohar*	Okinawa	0.018	0.018		-	0.018	0.006	<LOQ	<LOQ	-	-	0.006
10	NIHS-FE20105	*L. bohar*	Okinawa	<LOQ	0.005	0.003	-	0.010	<LOQ	0.004	0.006	-	-	0.006
11	NIHS-FE20106	*L. bohar*	Okinawa	<LOQ	-	-	-	0.002	-	-	<LOD	-	-	<LOD
12	NIHS-FE20107	*L. bohar*	Okinawa	0.078	0.088		-	0.110	0.040	0.024	0.057	-	-	0.097
13	NIHS-FE20108	*L. monostigma*	Kagoshima	0.025	0.024		-	0.026	0.016	0.007	0.006	-	-	0.022
14	NIHS-FE20109	*L. monostigma*	Okinawa	0.014	0.009	0.008	-	0.016	0.007	0.004	0.005	-	-	0.012
15	NIHS-FE20110	*L. monostigma*	Okinawa	0.100	0.070		-	0.122	0.089	0.013	0.009	-	-	0.098
16	NIHS-FE20111	*V. louti*	Okinawa	0.208	0.160		-	0.220	0.105	0.054	0.156	-	-	0.261
17	NIHS-FE20112	*V. albimarginata*	Okinawa	0.090	0.082		-	0.086	0.067	0.087	0.093	-	-	0.160

-: not detected (no signal)
Biosensor: LOD=0.001 μg/kg, LOQ=0.005 μg/kg
Colorimetric ELISA: LOD=0.0004 μg/kg, LOQ=0.0012 μg/kg
Fluorescent ELISA: LOD=0.00004 μg/kg, LOQ=0.0001 μg/kg
CBA: LOQ=0.001 μg/kg
LC-MS/MS: LOD=0.002 μg/kg, LOQ=0.005 μg/kg

Table 2. Comparison of the performance parameters of the different analytical techniques for detecting Pacific CTXs.

Technique	Calibrant/sample amount	Sensitivity	Detection mechanism*	Measurement format	Training requirement	Cost	Potentially portable	Assay time[#]	Used in this work
MBA	High	Low	Toxicity	Serial	Low	Low	No	Long	No
RBA	Low	Medium	Toxicity	Parallel	Medium	Medium	No	Medium	No
CBA	Low	High	Toxicity	Parallel	High	Medium	No	Long	Yes
ELISA	Low	High	Immunochemistry	Parallel	Low	Medium	Yes	Medium	Yes
Biosensor	Low	High	Immunochemistry	Serial	Low	Medium	Yes	Short	Yes
LC-MS/MS	Medium	High	Mass/Structure	Serial	High	High	No	Medium	Yes

[#]CTXs extraction and purification not included.

Declaration of competing interest

There are no conflicts of interest to declare.

Acknowledgments

This work was supported by the Agencia Estatal de Investigación (AEI) and the Fondo Europeo de Desarrollo Regional (FEDER) through the CIGUASENSING (BIO2017-87946-C2-2-R) and the CELLECTRA (PID2020-112976RB-C21) projects and by the European Food Safety Authority through the EuroCigua project (GP/EFSA/AFSCO/2015/03). The authors acknowledge support from CERCA Programme of the Generalitat de Catalunya. The authors thank Prof. Takeshi Yasumoto (JFRL) for providing the CTXs reference materials to IRTA, OPU and NIHS, and purified CTXs to NIHS. The authors thank Prof. Richard J. Lewis (The University of Queensland) for providing purified CTX1B to IRTA.

Author contributions

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Takeshi Tsumuraya: Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Resources; Writing - original draft; Writing - review &editing.

Masahiro Hirama: Investigation; Resources; Writing - review &editing.

Jorge Diogène: Funding acquisition; Investigation; Project administration; Resources; Writing - review &editing.

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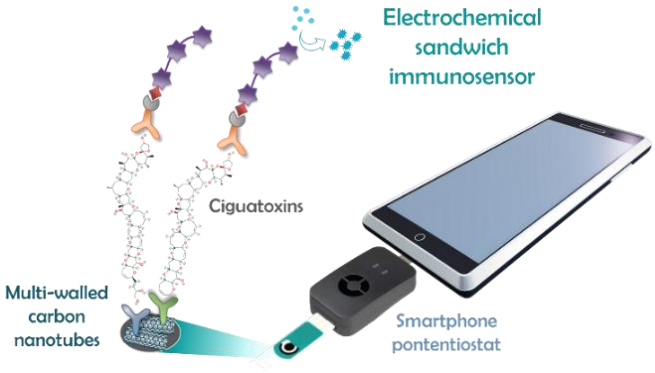
Graphical abstract



Supplementary information

Figure S1. Map with the fish collection sites.

Table S1. Weight, length and origin of the fishes.

	Code	Species	Origin	Weight (g)	Length (mm)	Origin
1	NIHS-FE20001	*L. bohar*	Okinawa	-	-	fisher
2	NIHS-FE20002	*L. bohar*	Okinawa	404	208	fisher
3	NIHS-FE20003	*L. monostigma*	Kagoshima	-	330	fisher
4	NIHS-FE20004	*V. louti*	Ehime	4,800	5,500	disapproved
5	NIHS-FE20113	*G. javanicus*	Fiji	1,845	855	fisher
6	NIHS-FE20101	*L. bohar*	Okinawa	-	-	fisher
7	NIHS-FE20102	*L. bohar*	Wakayama	12,000	-	disapproved
8	NIHS-FE20103	*L. bohar*	Wakayama	11,000	-	disapproved
9	NIHS-FE20104	*L. bohar*	Okinawa	2,000	390	fisher
10	NIHS-FE20105	*L. bohar*	Okinawa	477	250	fisher
11	NIHS-FE20106	*L. bohar*	Okinawa	1,028	294	fisher
12	NIHS-FE20107	*L. bohar*	Okinawa	-	-	fisher
13	NIHS-FE20108	*L. monostigma*	Kagoshima	-	-	fisher
14	NIHS-FE20109	*L. monostigma*	Okinawa	-	-	fisher
15	NIHS-FE20110	*L. monostigma*	Okinawa	-	-	fisher
16	NIHS-FE20111	*V. louti*	Okinawa	3,040	496	fisher
17	NIHS-FE20112	*V. albimarginata*	Okinawa	-	-	fisher