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Chapter 1

CIGUATERA SHELLFISH POISONING (CSP): A NEW ECOTOXICOLOGICAL PHENOMENON FROM CYANOBACTERIA TO HUMANS VIA GIANT CLAMS

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ABSTRACT

Giant clams, although frequently consumed in the South Pacific, are rarely incriminated in human intoxications. However, they are sometimes involved in strong and atypical Ciguatera Fish Poisoning (CFP) incidents, classically known to result from the ingestion of toxin-containing tropical fish associated with coral reefs. Nowadays, CFP risk assessment and management are still based exclusively on the monitoring of one known causative agent, the benthic dinoflagellate *Gambierdiscus*. Indeed, in certain favourable conditions, ciguatoxins (CTXs), a family of polyether neurotoxins produced by this microalga, can potentially accumulate up the food chain to reach high concentrations in fish specimens at the upper trophic levels, thus exposing *consumers* to serious health *risks*.

Following recent ecotoxicological studies conducted in the islands of Lifou (Loyalty Islands, New Caledonia), Raivavae (Austral Archipelago, French Polynesia) and Emao (Republic of Vanuatu), we demonstrated the link between the presence of cyanobacterial blooms and the occurrence of poisonings incidents by giant clams and/or fish from lower trophic levels, resulting in both ciguatera-like and paralysing symptoms. Toxicological studies using the mouse bioassay, the neuroblastoma cell-based assay and the receptor-binding assay showed the presence of CTXs-like and paralysing toxins both in cyanobacteria and the molluscs collected from contaminated locations.

During cyanobacterial blooms, filter-feeding bivalve molluscs such as giant clams are likely to become contaminated, thus providing a new link for the transfer of cyanotoxins to upper trophic levels including humans. The name “Ciguatera Shellfish Poisoning” (CSP) is proposed to designate this newly deciphered ecotoxicological phenomenon. The symptoms of this particular poisoning include the characteristics of CFP (reversal of sensations, itching and bradycardia) associated with additional symptoms like the burning of the mouth and the throat that appear very quickly and are followed by severe paralysis.

We recommend that future field monitoring programs of toxic organisms include the survey of toxic cyanobacterial blooms concurrently with other toxic microalgae, toward a more effective management of tropical seafood poisonings.

Keywords: giant clams, cyanobacteria, marine toxins, ciguatera fish poisoning, ciguatera shellfish poisoning.

1. INTRODUCTION

Cyanobacteria occupy a large range of marine, freshwater and terrestrial habitats. Widespread in marine ecosystems, they play a major role in the fixation of the atmospheric carbon and nitrogen. Under certain environmental conditions, some pelagic and benthic cyanobacteria can proliferate over wide areas. For example, *Lyngbya majuscula* (Oscillatoriales) that grows loosely attached to seagrass, sand and rocky outcrops, may sometimes form blooms occupying a surface area of 10 - 30 km^2 (Albert *et al*., 2005). Like freshwater cyanobacteria, cyanobacteria proliferating in marine environments are an important source of structurally diverse bioactive secondary metabolites (Tan, 2007). Some of these compounds show a strong cytotoxicity (lyngbyatoxins, lyngbyabellins, aplysiatoxins, dolastatins, curacin, aurilide, etc.), some are also involved in swimmers' dermatitis (aplysiatoxins and lyngbyatoxins)(Osborne *et al*., 2001). Three neurotoxic lipopeptides that are strongly ichthyotoxic were isolatedfrom *L. majuscula* (Oscillatoriales). These compounds acting on the voltage-sensitive sodium channels (VSSC) were characterized as potent activator such as antillatoxin A (Li *et al*., 2001) or blockers such as kalkitoxin (Lepage *et al.,* 2005) and jamaïcamide A, B and C (Edwards *et al.,* 2004).

Although phylogenetically distant from dinoflagellates, cyanobacteria have already been suspected as a likely source of ciguatoxins-like compounds. As early as the 1950s, Randall assumed that a benthic organism, most likely a blue-green alga, was the source of the toxin (Randall, 1958). Based on the presence of the benthic cyanobacterium *L. majuscula* in the guts of a large number of poisonous fishes, Halstead (1967) hypothesized that these cyanobacteria might serve as a primary source of ciguatoxins (CTXs) or their progenitors. Indeed, typical Ciguatera Fish Poisoning (CFP) signs of intoxication (quiescence, piloerection, diarrhoea, lachrymation, cyanosis, dyspnoea, convulsive spasms and death by respiratory failure within 24 hours) have been demonstrated in mice injected intraperitoneally with extracts of both *Trichodesmiumerythraeum* and molluscs (Hahn and Capra, 1992; Endean *et al*., 1993).

CFP is prevalent *worldwide* in subtropical and tropical regions (50,000 to 100,000 cases per year) (Fleming *et al*., 2006), and is caused by the ingestion of tropical coral reef fish contaminated with CTXs produced by thedinoflagellates *Gambierdiscus* (Gonyaulacales)(Holmes *et al.,* 1991; Babinchak *et al*., 1994; Yasumoto, 2005; Litaker *et al*., 2010). CTXs act on VSSC present on most excitable and some non-excitable cells (Nicholson and Lewis, 2006). CTXs bind directly to site 5 on the α-subunit of VSSC (Poli *et*

al., 1986), causing them to open at normal cell resting membrane potential. The resulting influx of sodium ions (Na^+) induces membrane depolarization (Benoit *et al.,* 1986) and causes spontaneous firing in a variety of nerve fibers (Benoit *et al.,*1996). CFP alone is responsible for more cases of human poisonings than all other marine toxins combined. Although this phenomenon is also endemic in the Caribbean and the Indian Ocean, the most affected regions remain the Pacific Island countries and territories where fishing is a subsistence resource of great *nutritional*, economical and cultural importance (Laurent *et al.,* 2005). Indeed, in these regions, seafood resources both constitute the main source of protein and a substantial source of income for local communities. High consumption of fish and seafood, obtained from the wild or farmed, characterizes the coastal tropical communities, which are marked and supported by traditional fishery. Consequently, outbreaks of poisoning have a significant negative impact on human health, but also on the local economy and social structure. It modifies the behaviour of individual and entire populations that looses a major component of diet by the fear of becoming intoxicated. In areas strongly affected by CFP, this situation may indirectly lead to enforced dietary transition, totally transforming the traditional nutritional systems, thus favouring the expansion of civilization diseases such as obesity, cardiovascular diseases and diabetes (Chinain *et al.,* 2010).Giant clam toxicity has been to date poorly documented compared to other incidents of seafood and fish poisoning. Besides the recently reported cases in Lifou (Laurent *et al.,* 2008), 33 cases of human poisonings, of which two were fatal, had been recorded in Bora Bora Island (French Polynesia) poisoned by *Tridacna maxima* (*Tridacnidae*) collected in a ciguateric area (Bagnis, 1967); Bagnis emphasised the presence of bluish alga covering the giant clams in the toxic area. Banner (1967) could collect some specimens and confirm the toxicity of their viscera and mantles in a mongoose bioassay. Based on preliminary chemical analysis, he detected two toxins, one soluble in water and the other one in organic solvents. The water-soluble toxin showed moderate anticholinesterase activity. In addition, an epidemiological survey of CFP conducted in 1974 in the Gambier Islands (French Polynesia), established that 4% of all cases of poisoning were due to giant clam consumption (Bagnis, 1974). Furthermore, Kanno *et al.* (1976) showed that *Tridacna marmorata* contained an appreciable amount of a water-soluble toxin and also a remarkable toxicity in the acetone-soluble fraction in the hepatopancreas of *T. maxima*. In other studies, paralytic shellfish toxins (PSTs) have been detected in *Tridacna crocea* and linked to *Pyrodinium bahamense* var. *compressa,* dinoflagellate blooms in Palau (Caroline Islands) (Harada *et al.,*1982; Hwang,

2003). More recently, others molluscs, such as *Trochus* sp. (*Trochidae*), have been incriminated in fatal human poisonings in New Caledonia (Angibaud *et al.,* 2000).Recently, studies conducted in Lifou island (Loyalty Islands, New Caledonia), where the native inhabitants have been heavily affected by the ecotoxicological phenomenon of CFP from 2001 to 2005 and by giant clam poisonings, revealed for the first time the potential toxicity of marine benthic cyanobacteria (*Hydrocoleum* spp.) (Laurent *et al.,* 2008). These Oscillatoriales were found to produce lipid-soluble compounds similar to ciguatoxins (CTX-like compounds), as well as water-soluble paralysing toxins such as PSTs and/or anatoxin-a (ANTX) and homoanatoxin-a (HANTX) (Laurent *et al.,* 2008). This original report suggested a possible relationship between human seafood intoxications and neurotoxins-producing marine benthic cyanobacteria. The production of ANTX and HANTX by *Hydrocoleum* spp. was further confirmed using gas chromatography coupled to mass spectrometry (GC-MS) analysis (Mejean *et al.,* 2010). As a result, it was hypothesized: i) that these different toxins can subsequently accumulate in the surrounding filter-feeding shellfish such as giant clams, thus having potential to cause serious ciguatera-like symptoms and ii) that these molluscs, most often consumed and cultivated in the Pacific, could provide a new link in the food chain transfer of toxins similar to CFP, thus constituting a new ecotoxicological phenomenon that we could call Ciguatera Shellfish Poisoning or CSP.To confirm the relationship between the proliferation of toxic marine cyanobacteria and giant clams toxicity, three missions per year were run in Lifou during the three last years, two field missions were organized in Emao (Republic of Vanuatu) in 2009 and several field missions were conducted in Raivavae (Australes Archipelago, French Polynesia) from April 2007 to September 2010. Here, we present the epidemiological surveys and the results relative to the toxicity of cyanobacteria and giant clams collected in these three locations, in order to decipher the origin of these uncommon incidents of giant clam poisoning.

2. MATERIALS AND METHODS

2.1. Epidemiological Surveys

2.1.1. Lifou (Hunëtë tribe)

The tribe of Hunëtë, located in the Santal bay at the northern part of Lifou (Loyalty Islands, New Caledonia) (Figure 1), populated by 300 inhabitants has

experienced a serious episode of CFP (Laurent *et al.,* 2008). The epidemiological survey in Lifou began in 2005 with interviews of 35 native residents of the tribe that were affected by seafood poisoning over the last 5 years. Epidemiological and clinical forms were designed according to a field reference guidebook on CFP, co-edited by the Secretariat of the Pacific Community (SPC) and IRD (Laurent *et al.,* 2005). This questionnaire was divided into four parts: (i) information about the person in care of the patient and filling out the form (doctor, health worker, etc.); (ii) information about the patient (name, age, sex, etc.); (iii) information about the seafood product(s) that caused the poisoning (type, amount and origin), and (iv) patient's medical history (clinical picture and parameters).In addition, the chief of the fishermen clan was also questioned about their fishing habits, the existence of high-risk areas and the natural and anthropogenic environmental changes during these last years. Several missions (10) were conducted between 2007 and 2010 during which the cyanobacterial blooms were observed and collected. To facilitate the ecotoxicological observations, 6 transects were positioned perpendicular to the coast each with three collection points. Three transects were placed in the area deemed toxic and three in the area considered safe. The *Hydrocoleum* blooms covering dead coral were collected by scraping while cyanobacteria covering sand were collected manually *in situ*. Samples of giant clams (mainly *Tridacna* sp.) and parrotfishes (mainly *Scarus schlegeli* and *S. rivulatus*) were collected each year, during the warm seasons, to analyze their toxicity.

2.1.2. Emao

Emao is a very small island (3.4 km of width) situated NE of Efate (Republic of Vanuatu). Although there are six villages on Emao, only Lausake village, located on the southeastern side, was affected by fish and giant clam poisonings (Figure 2). The aims of the first mission in May 2009 were i) to contact communities affected by the fish poisoning epidemic and collect historic information, ii) to deliver the up to date information about ciguatera and marine-related illnesses and improve the community's understanding of them, and iii) to obtain information about the extent of the infected reef area through field inspection. During the second mission in November 2009, dinoflagellates, cyanobacteria, fishes (mainly *Acanthuridae*, *Scaridae* and *Lethrinidae*), and giant clams (mainly *Hippopus hippopus*) were collected in front of the affected village Lausake as well as of Wiana, a neighbouring village safe from CFP, for comparative toxicological studies.

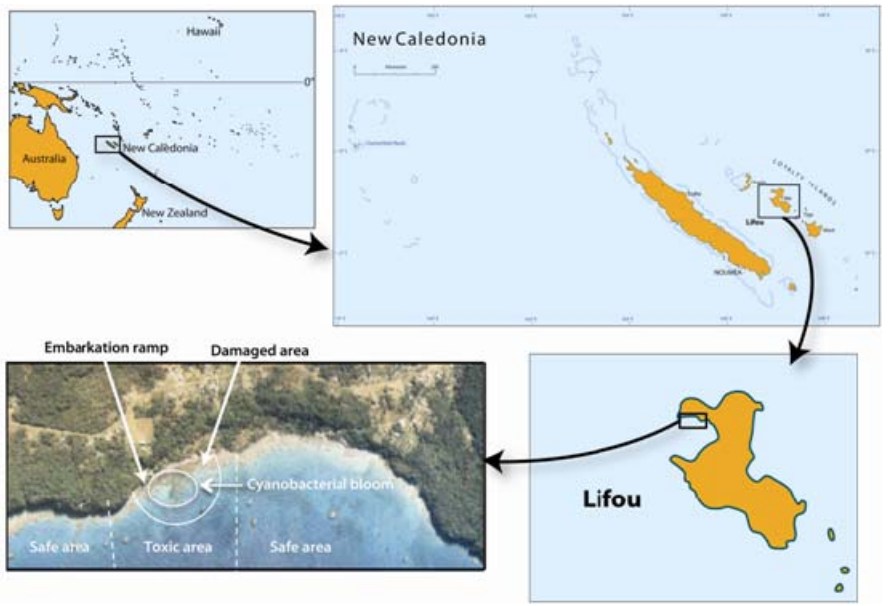

Figure 1. Study area in Lifou (Loyalty islands, New Caledonia)(Picture source: DITTT NC Government).

2.1.3. Raivavae

Raivavae is a small island (8.5 km long) in Austral Archipelago, French Polynesia, located about 700 km SE of Tahiti. This island is highly affected by CFP and cases of poisoning due to consumption of giant clams have also been reported in the recent years. Five consecutive missions were conducted in Raivavae: i) during the hot season (April 2007, February 2009 and February 2010) with an average seawater temperature (AWT) of 26°C, ii) during the cold season, (September 2007, AWT= 23°C) and iii) during an intermediate season (May 2008, AWT = 25°C).

Interviews with the local population about seafood poisonings they experienced in the past were conducted during the initial mission. In order to map the ciguatoxic risk at this time, fishes from all types of diet, herbivorous, omnivorous and carnivorous, were collected at various locations around the island (Chinain *et al*., 2010). The obtained information combined with our underwater observations around the island have allowed us to designate 4 particular study sites, where the evolution of cyanobacterial mat and the toxicity of giant-clam (mainly *Tridacna* sp.) could be monitored during each mission (Figure 3).

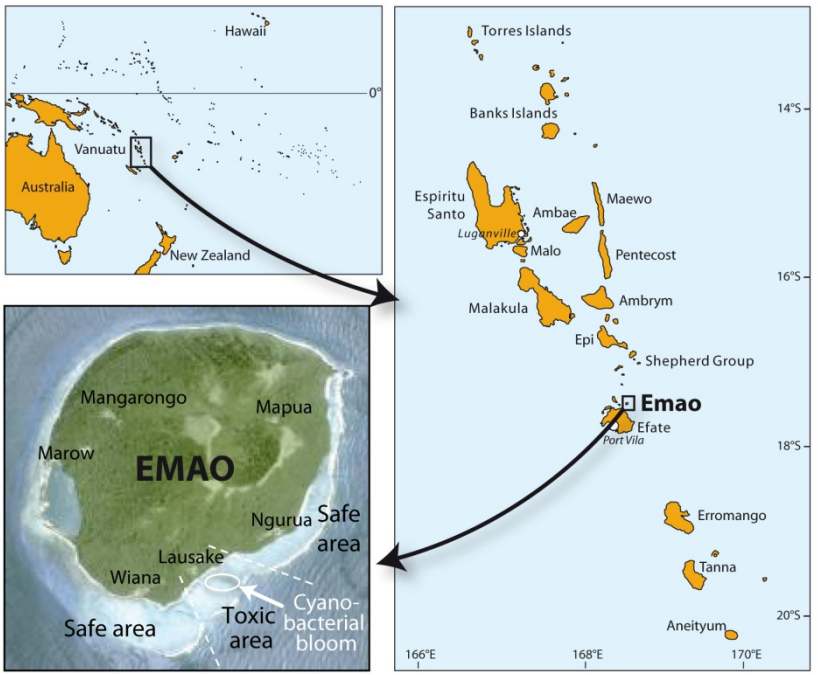

Figure 2. Study area of Emao (Republic of Vanuatu)(Picture source: Google Earth).

2.2. Examination of Toxic Cyanobacteria

2.2.1. Sampling

Samples of mat-forming cyanobacteria were collected during each of the missions. Cyanobacterial samples of Lifou were collected by manual transfer into plastic bags. In subsequent samplings in Emao and Raivavae, samples were collected using an underwater suction device to increase the biomass yield. Samples were left for several hours in the light at room temperatureto allow motile cyanobacterial trichomes to move phototactically and aggregate. The resulting cyanobacterial pellets were stored at 20°C until further extraction for toxicity tests.

Cyanobacterial trichomes subsampled for morphological and genetic identification were fixed in 5% formaldehyde and ethanol respectively.

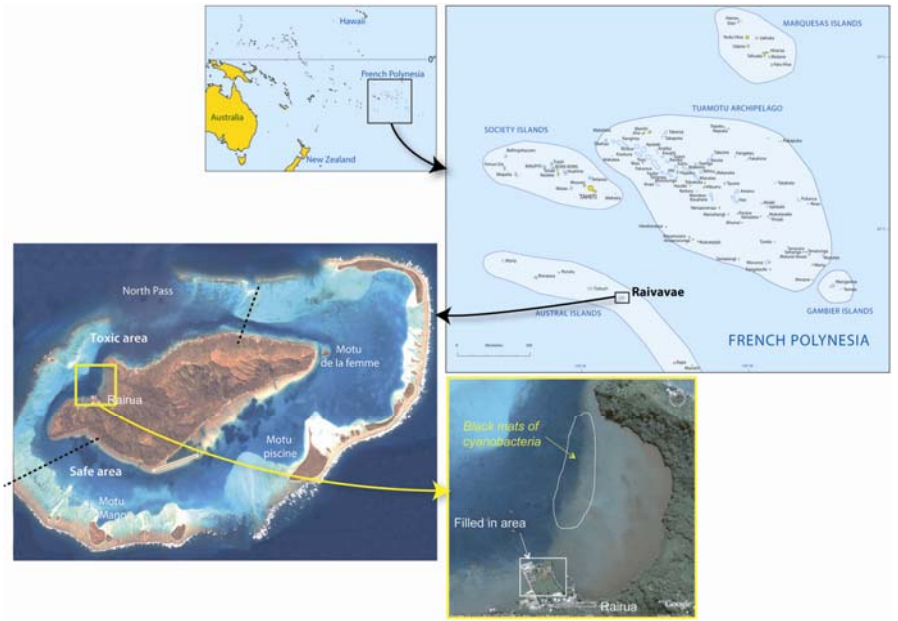

Figure 3. Study area of Raivavae (Australes archipel, French Polynesia)(Picture source: Google Earth).

2.2.2. Microscopy and Taxonomic Identification of Cyanobacteria

Phenotype analysis has been performed and photodocumented using Zeiss Universiam light microscope and digital camera and a camera lucida in-scale projection of the same company. The in-scale drawings were scanned, measured and statistically evaluatedusing Sigma-Scan software (Sausalito, Ca).The parallel samples preserved in 70% ethanol were saved for DNA analysis planned to be carried out in a separate study.

2.3. Extraction and Preparation of Toxic Extracts

2.3.1. Cyanobacteria

The extraction procedure for cyanobacteria was adapted from the method described by Chinain *et al.* (1999) (figure 4). Briefly, in order to characterize the lipid-soluble toxin(s), freeze-dried powders of cyanobacteria were extracted with 2 L of methanol per kg of crude material and further partitioned between 1 L of dichloromethane and 2 x 500 mL of 60% aqueous methanol

per kg. The dichloromethane phase was dried in a rotary evaporator and defatted by a second solvent partition using 200 mL of cyclohexane and 100 mL of 80% aqueous methanol. The final methanolic phase (lipid-soluble extract) was evaporated and its toxicity was evaluated.

The lipid-soluble extracts were screened for the presence of CTX-like compounds by mouse bioassay (MBA), neuroblastoma cytotoxicity assay (NCA) and receptor binding assay (RBA), while the toxicity of water-soluble extracts was estimated by MBA.

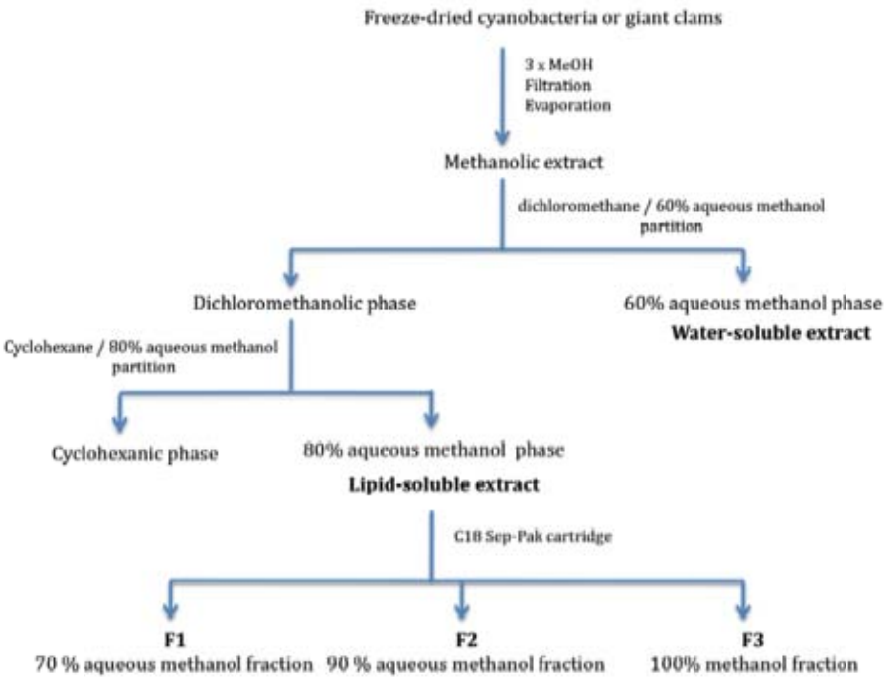

Figure 4. Fractionating method of cyanobacterial and giant clams methanolic extracts.

For the toxicity screening via NCA and RBA, 2 aliquots of 1 or 2 mg of lipid-soluble extract were sampled and processed through a C18 Sep-Pak cartridge. Briefly, Sep-Pak C18 cartridges (Waters®) were pre-conditioned with 7 mL aqueous methanol (70:30) before loading 7 mL of crude extracts in the presence of 3 mL of distilled water. The column was washed with 2 x 7 mL aqueous methanol (70:30) (fraction F1) before elution with 7 mL aqueous methanol (90:10) (fraction F2).

Then the column was washed with MeOH 100% to give a third fraction F3. The three fractions were subsequently dried under vacuum for 150 min at 60°C in a SpeedVac AES1010 concentrator, and stored at 4°C until tested by NCA and RBA. CTXs should appear in fraction F2.

To further assess the potential occurrence of PSTs (Negri and Jones, 1995) or ANTX (Araoz *et al*., 2005) in these samples, the residual crudecyanobacterial methanolic extract was treated with acidified methanol (0.05 M acetic acid) then partitioned as the first methanol extract. These water-soluble acidic fractions were also screened for paralysing toxins using MBA.

2.3.2. Giant Clams

At each sampling site, 10 specimens of giant clams were collected, measured and weighted, and all their flesh pooled prior to grinding with a stomacher 441. For each pool, extracts were obtained by soaking of 1 kg of fresh tissue twice in 2 L of methanol under sonication for 4 h. The resulting methanolic phase was filtered through a Buchner® filter and dried under vacuum.

Successive solvent partitions (dichloromethane/60% aqueous methanol and cyclohexane/80% aqueous methanol) were applied to the dry methanolic residue following the procedure previously described for cyanobacterial samples. The final methanolic phase (lipid-soluble extract) was evaporated and its toxicity was evaluated by MBA. For the toxicity screening via NCA and RBA, 2 aliquots equivalent to 0.2, 0.5, 1 or 2 g of flesh were sampled and processed through a C_{18} Sep-Pak® cartridge as described for the cyanobacteria. The toxicity of the 60% aqueous methanol phases (water soluble extract) was estimated by MBA.

As for cyanobacteria, some giant clam samples were extracted with acidic methanol (0.05 M acetic acid) to screen for the potential occurrence of PSTs or ANTX.

2.3.3. Fishes

Each fish was photographed, measured and weighted, and its flesh cut as fillets and stored at – 20°C until further CTX extraction. Fish extracts intended for RBA were prepared following the procedure described by Darius *et al.* (2007). For each individual, 2 x 5 g of flesh was homogenized in a Stomacher 441waste disposal unit for 2 min, and re-suspended in 5 mL methanol under sonication for 2 h, prior to incubation overnight. After centrifugation at 850 *g* for 5 min, the supernatant supposed to contain CTXs was subjected to subsequent purification using C_{18} Sep-Pak® cartridge (Waters)as described for

the cyanobacteria. The resulting extract was further dried under vacuum for 3 h at 60°C in a SpeedVac concentrator, and stored at 4°C until tested for its toxicity.

2.4. Mouse Bioassay (MBA)

All animal experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) using 19 - 21 g OF1 mice of either sex (Charles River Laboratories, France). Accordingly, all efforts were made to minimise animal sufferings and a minimum number of mice was used with food and water provided *ad libitum*. Finally, mice were sacrificed if they survived more than the observation period post-extract injection. Groups of two animals were intraperitoneally (i.p.) administered a single dose of lipid-soluble extract dissolved in 250 µL of a 0.9% saline solution containing 0.1% Tween 60 (detergent used as emulsifier). Mice were observed over 24 h and signs of intoxication and time to death recorded. Fractions were considered non-toxic if injection of a maximal dose was not lethal. Total toxicity of lipid-soluble extracts was deduced from the mean survival time of injected mice, based on dose/survival time graphs previously established using CTXs purified from wild *Gambierdiscus*. Total lethality was expressed in mouse units (MU).mg^{-1} of extract of cyanobacteria, or MU.g^{-1} of flesh of giant clam. One MU is defined as the i.p. LD_{50} dose for a 20 g mouse over 24 h.

Similarly, three different doses of water-soluble extracts were emulsified in the diluent and injected i.p. in mice in duplicate. Symptoms and behavioural changes were observed over a 48 h period.

As controls, some mice were given the diluent only under similar conditions to those used for the testing of both lipid- and water-soluble extracts.

2.5. Neuroblastoma Cytotoxicity Assay (NCA)

Cyanobacteria and giant clams lipid-soluble extracts were tested for their toxicity using NCA, a test developed by Manger (1995) to detect sodium channel-enhancing marine toxins. Briefly, mouse neuroblastoma cells (Neuro-2A, CCL-131 line obtained from ATCC) were seeded into 96-well culture plates at a density of 2.5 x 10^5 cells mL^{-1} and left to incubate for 24 h. Cells

were then treated with ouabain (O) and veratridine (V) and further incubated 14 h in presence or absence of lipid-soluble extracts. The concentrations of O and V were respectively 500 µM and 50 µM to obtain about 80 % of cell viability. In order to check for potential matrix effect or the presence of bioactive compounds other than sodium channel toxins, parallel experiments were conducted without ouabain/veratridine cocktail. After treatment, the media was removed and cell viability was measured using the tetrazolium salt (MTT;3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Mossman, 1983). The absorbance of its reduced form produced by metabolically active cells was measured at 490 nm using a microplate-reader (ELX 800, Bio-Tek Instruments, Inc., Fisher Scientific, France). The cytotoxicity of lipid-soluble extracts was assessed based on EC_{50} values expressed in $\mu g.mL^{-1}$ of extract, where EC_{50} is defined as the effective concentration of extract capable of inducing 50% of mortality in cells. Sigmoidal curve fitting and EC_{50} calculations were performed using GraphPad Prism version 4.01 (*GraphPad Software*, San Diego, California, USA). Curves with a coefficient of nonlinear regression (R^2) > 0.9 were considered.

2.6. Receptor Binding Assay (RBA)

RBA has been considered as a very promising alternative to MBA to detect and quantify specifically CTXs in fish (Van Dolah *et al*., 1994; Bottein Dechraoui *et al*., 2005). Basically, the RBA measures the residual binding of a constant concentration of tritiated brevetoxin ([^{3}H]PbTx-3) to its specific receptor on the sodium channel of rat brain cells in the presence of increasing amount of unlabeled toxin contained in lipid-soluble extracts. Rat brain synaptosomes were prepared as previously described by Dechraoui *et al.* (1999). Protein concentration of synaptosomal preparations was determined by assaying aliquots in duplicate using the Bradford protein assay with serum albumin (BSA) as a standard. A final protein concentration of 60-90 µg/mL was used giving no more than 10% of the total radioactivity. The RBA was performed in a test tube format with [^{3}H]PbTx-3 (0.90 nM), following the protocol of Darius *et al.* (2007). Non-specific binding was measured in the presence of saturating concentration of PbTx-3 (0.67 µM) and subtracted from the total binding to yield specific binding. P-CTX-3C obtained from a clonal culture of *Gambierdiscus polynesiensis*, was used as an internal standard for sample calibration and yielded an IC_{50} value of 0.62 $ng.mL^{-1}$(Chinain *et al.,* 1999; Darius *et al.,* 2007). For all samples, 2 aliquots were tested and

measured in parallel with a range of 8 dilutions in duplicate. Unknown sample concentrations were calculated from the IC_{50} values, i.e. concentration of sample ($mg.mL^{-1}$ for cyanobacteria and $g.mL^{-1}$ giant clam and fish samples), which causes 50% inhibition, determined using GraphPad Prism version 4.01. Then, RBA toxicity values were determined from IC_{50} values of cyanobacterial, giant clam and fish lipid-soluble extracts, using the following formulas: RBA_{cyano} = (0.62 $ng.mL^{-1}$/y $mg.mL^{-1}$) where 0.62 is the IC_{50} of P-CTX-3C and y the IC_{50} value of cyanobacterial extracts; RBA_{clam} = (0.62 $ng.mL^{-1}$/z $g.mL^{-1}$) where z represents the IC_{50} value of giant clam extracts; RBA_{fish} = (0.62 $ng.mL^{-1}$/x $g.mL^{-1}$) where x represents the IC_{50} value of fish extracts. RBA_{cyano} was expressed in ng P-CTX-3C eqv.mg^{-1} of dried cyanobacterial extract and RBA_{clam} and RBA_{fish} were in ng P-CTX-3C eqv.g^{-1} of giant clam or fish flesh, respectively. To make comparisons with former studies easier, these values can also be converted into P-CTX-1B equivalents using the following formula; RBA_{1BEq} = (RBA values in P-CTX-3C eqv / 2.31). Quality control of the assay was performed by testing each receptor binding assay with a toxic extract of *Gambierdiscus polynesiensis* with a known concentration of 3.1 pg P-CTX-3C equivalent (n = 2).

3. RESULTS

3.1. Population Interviews

3.1.1. Lifou

Inhabitants have delimited a toxic area intuitively confirmed by a traditional prohibition of fishing by the chief of the fishermen clan to avoid more intoxication. From the 35 cases of poisonings, 30 were induced by seafood harvested from the toxic area, with 2 by a giant clam (*Tridacna* sp.), more than a half (23) by herbivorous or grazer fishes and 6 by molluscivorous fishes. This implication of herbivorous and molluscivorous fishes was particularly disturbing as these fishes are widely consumed because of their safety reputation in New Caledonia.

For one third of the cases including poisonings by giant clams, the symptoms began by a strong alteration to taste and a burning sensation on the tongue and the throat immediately after the meal.

These symptoms are not common of CFP. In contrast, as in typical CFP all patients suffer from gastrointestinal disorders, general fatigue, pain in the limbs and joints, reversal of hot and cold sensation as well as a tingling

sensation upon contact with water. However, the severity of the cardiovascular symptoms and serious problems of paralysis caused one third of the victims to be hospitalized, which is far greater than usually encountered in New Caledonia or in South Pacific.

The inhabitants of this tribe have experienced numerous CFP episodes in their lives, which they claimed to have successfully treated with indigenous herbal medicine commonly used in South Pacific (Kumar-Roine *et al*., 2011). However, during the recent episodes, although all victims used of commonly accepted traditional treatments, the majority reported the lack of effectiveness of traditional treatment.

3.1.2. Emao

During the first mission, about 40 people were interviewed. Each of them had been poisoned at least once by fish, shellfish or both. Presently, only tuna can be eaten without risk for this population but catching tuna requires a boat and engine, which most villagers do not have. Villagers noted differences in the symptoms from eating toxic fish and toxic shellfish. The symptoms associated with fish were typical of CFP: nausea, diarrhoea, joint pain, general fatigue, and hot-cold sensory reversals. With shellfish, symptoms appeared very quickly as in Lifou: tingling lips and burning sensations in the mouth, followed by gastrointestinal problems that occur within the first hour, and neurological symptoms that lasted for several weeks.

3.1.3. Raivavae

There is currently a transition in feeding habits of the Raivavae's population (905 inhabitants in 2007). The traditional consumption of fishes from the lagoon is avoided to minimize the risk of contracting CFP, instead canned food or chicken is supplemented instead (Dewailly *et al*., 2008). The CFP phenomenon is present in a large part of the island and appears to have emerged following the construction of the Northwest embankment as a part of a small port area and the blasting of many coral blocks to secure the channel from this area to North pass (Figure 3). The export of giant clams to Tahiti (Society Islands, French Polynesia) constitutes one of the island's important economic resources (25t/year). These clams are mainly harvested in the Southwest part of the lagoon (Motu Mano), but several specimens collected for personal consumption in the Rairua bay (near the port) have caused poisoning.

Two islanders previously poisoned by giant clams were identified and interviewed. In both cases, the implicated giant clams were collected in the bay at the East of the embankment, in front of the Rairua village. One of them

reported having suffered from a very severe itching during two years. A dozen medical visits followed by various antihistaminic treatments produced no significant relief. Two years after the poisoning, neurological investigation, at the central hospital in Papeete (Tahiti), revealed signs of neuropathy of the lower limbs. The second one, who consumed two giant clams, said having felt a strange taste in the mouth, then experienced blurred vision and loss of balance followed by diarrhoea, myalgia, a feeling of paralysis and laboured breathing. Unable to walk, he was taken to hospital and kept under observation for two days due to a very low arterial tension. These symptoms were associated with itching for one month, tingling in the hands and legs and the hot-cold temperature sensation reversals, all symptoms characteristic of CFP. It is noteworthy that these symptoms were very similar to those experienced by the patient severely poisoned during the CFP-like outbreak in Lifou (Laurent *et al*., 2008).During the interviews, the inhabitants revealed that, owing to their fishing habits, seafood consumption and poisoning past experiences, they have delimited a "high-risk area" within the lagoon lying between two borders located at the Southwest and the Northeast of the island (Figure 3), from which the harvesting of seafood is not recommended.

3.2. Environmental Observations

In all missions to Lifou, Emao and Raivavae, very few cells of *Gambierdiscus* or other dinoflagellates, *Ostreopsis* or *Prorocentrum* were observed in our samples, instead the areas were always coated by mats produced by benthic cyanobacteria.

3.2.1. Lifou

The area designated as toxic by the Hunëtë tribe extends 500 m along the coast and to about 50 m offshore, to the outer margins of the reef platform (Figure 1). This area was affected by the construction of an access road and an embarkation ramp in 1990, making a large break into the calcareous ridge of an elevated fossil coral reef. The bitumen-surfaced road was subsequently destroyed by heavy rains during the passage of a hurricane in 2003, and part of this material was deposited over the reef flat.

During past three years (2007 to 2009), 10 missions were conducted and a succession of cyanobacterial blooms of filamentous cyanobacteria (Oscillatoriales) (Figure 5) dominated by *Hydrocoleum glutinosum* Gomont (Figure 6A-B), and *H. lyngbyaceum* Kützing, Both populations were

characterized by short 2-5 µm long cells, by straight and shortly attenuated trichome ends with capitated, hourglass-shaped end cells, covered by a thickened cell wall (calyptra) (Laurent *et al.,* 2008), but differed in size, each population confined to a relatively narrow range of cell width: *H. glutinosum* with trichomes 18.88 ± 0.94 µm wide (expressed as mean ± standard deviation) and the significantly narrower *H. lyngbyaceum* 11.52 ± 0.79 µm wide. Another common filamentous cyanobacterium forming colonies varying in shapes and coloration was *Phormidium laysanense* Lemmermann (Figure 6C-D), characterized by calyptrate terminal cells and long trichome cells, 6.25 ± 0.7 µm wide and 6.46 ± 1.93 µm long.

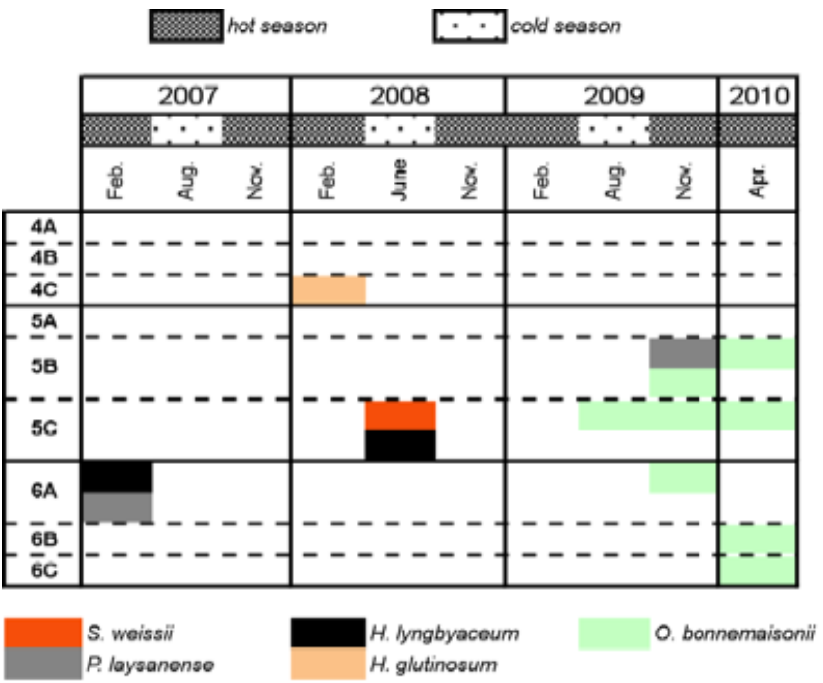

Figure 5. Succession of cyanobacteria (Oscillatoriales) blooms between 2007 and 2010 along the three transects (4, 5, 6) of the area deemed toxic by the inhabitants of Hunëtë (Lifou).

Large areas of orange colored loose mats were dominated by *Spirulina weissii* Drouet (Figure 6E-F) which was observed repeatedly within the toxic area.It is an unusually large *Spirulina* with trichomes 4.50 ± 0.37 µm wide,

forming 10.0 ± 0.62 µm wide compacted coils. The bloom of *O. bonnemaisonii* Crouan ex Gomont (Figure 6G-H), with wide flexible trichome cells 21.34 ± 1.03 µm wide and 4.65 ± 1.34 µm long.These mats were observed regularly in the toxic area, but also extended in the nearest transect of the «safe» area.

Figure 6. Marine benthic cyanobacteria associated with seafood poisonings in Lifou, Loyalty Islands and New Caledonia: field view of the mats (left) and microscopic view of the predominant organism (right). Scale bars are 20 µm long in all photomicrographs. A – B. *Hydrocoleum glutinosum*, June, 2008; C – D. *Phormidium laysanense*, November, 2009; E – F. *Spirulina weissi*, May, 2006; G – H. *Oscillatoria* cf. *bonnemaisonii,* November, 2009.

It is interesting to note the total absence of macroalgae and of sea cucumbers, *Stichopus chloronotus* (*Stichopodidae*); these echinoderms being present in large amount in the neighbouring safe area.

3.2.2. Emao

According to the population of Emao, the toxic area covers the reef adjacent to Lausake village, from the beach outward to the barrier reef (ca 1,000 m off shore).

The reef fish became toxic 10 years ago while giant clams, *Trochus* and other gastropods became toxic about three to four years ago. The reef area consisted mostly of *Acropora* spp., most of which dead and covered with carpets of filamentous cyanobacteria. In contrast, in Wiana village,which served as the control zone, presumed by the local population as non-toxic, the corals appeared healthy and no cyanobacterial mats were observed.

The observed cyanobacteria produced bright glue-green mats with upright filaments waving in the current, comprised by heterocystous cyanobacterium *Anabaena* sp. (Figure 7A-B). Common on Emao Island were also *H. glutinosum* (Figure 7C-D),with trichomes 18.67 ± 0.86 µm wide and *Lyngbya sordida*(Figure 7E-F), with trichome cells were 20.61 ± 1.39 µm wide and 7.9±1.82 µm long, which formed diverse mats of variable extent.

3.2.3. Raivavae

Based on our previous experiences in Lifou (Laurent *et al*., 2008), we organized and conducted five field missions to Raivavae between 2007 and 2010 in order to evaluate the evolution of the phenomenon there and to address the issue of seasonality of cyanobacterial bloom occurrences. Following underwater observations in the lagoon all around the island during the first mission, two sites were designated for a regular monitoring including taxonomic determination and the potential toxicity of the benthic cyanobacterial taxa and of giant clams: the area in front of the Rairua village on the West and the area around the “Motu de la femme” on the East coast of the island (Figure 3). As control sites, two locations were chosen: the “Motu Mano” in the Southwest, where the islanders harvest giant clams for marketing in Papeete and the “Motu Piscine” in the Southeast, where these bivalves are offered for tasting to tourists.

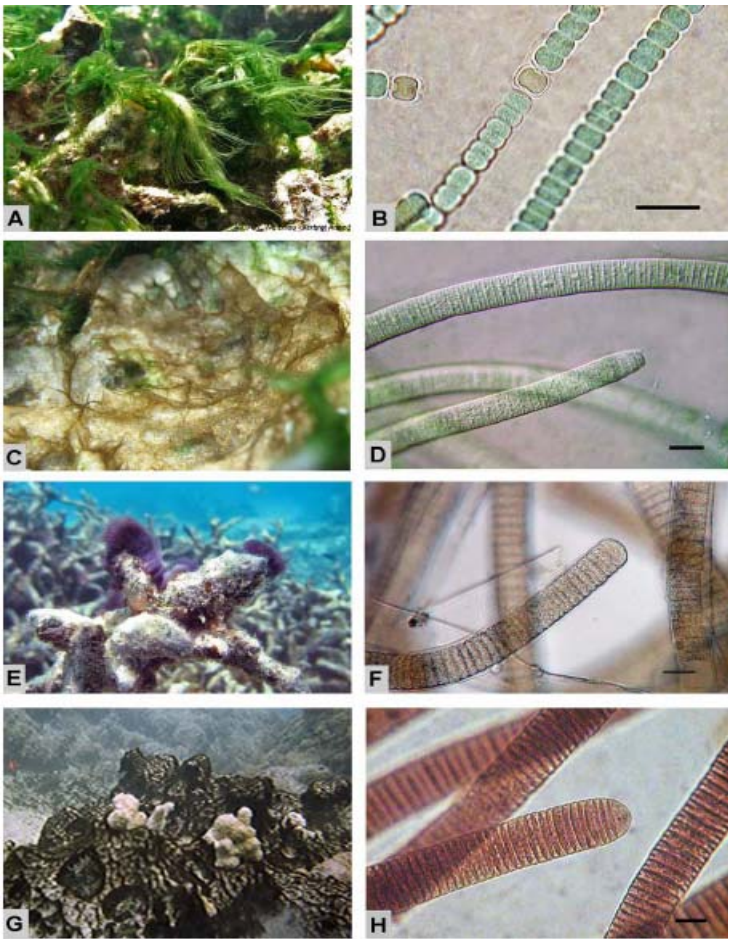

Figure 7. Marine benthic cyanobacteria associated with seafood poisoning on Emao, Republic of Vanuatu (A-F) and Raivavae, Australes, F.P. (G-H). Scale bars in all pictures are 20 µm long. A – B. *Anabaena* sp., Emao, November 2009; C – D. *Hydrocoleum glutinosum*, Emao, November 2009; E – F. *Lyngbya sordida*, Emao, November 2009; G – H. *Oscillatoria* cf. *bonnemaisonii*, Raivavae, April 2007.

3.2.3.1. Rairua Bay Area

In April 2007 (hot season), underwater observations revealed the presence of shiny black cyanobacterial mats covering primarily tufts of *Halimeda* (Chlorophyceae), lining the bottom of the bay at a depth of 2 to 5 m over an estimated surface of about 500 m long and 50 m wide (Figure 3). Microscopic observations of these cyanobacteria identified the principal constituent of the

mat as *O.bonnemaisonii*(Figure 7G-H). Trichomes were mostly irregularly bent, but also helically twisted (5 % in the sample), 24.17 ± 3.05 μm wide with short discoid cells 5.53 ± 1.28 (34)μm long.

Trichomes were briefly attenuated at the tips with terminal cells rounded without calyptra, slightly narrower and longer than the trichome cells 20.66 ± 1.80μm wide and 10.90 ± 2.41 μm long. The populationwas rich in phycoerythrin, with some variation in pigment density among them. In this area no dinoflagellates have been observed during the cyanobacterial bloom in April 2007.

In September 2007 (cold season), an almost complete disappearance of the cyanobacterial mats was noticed, leaving only in rare localized dark cyanobacterial patches, which were collected for toxicological analysis. In May 2008 (intermediate season), the bloom was also patchy, but with more numerous dark spots than in September. At this period and at the same site, an associated large bloom of *L. majuscula* was observed. In February 2009 and 2010, small mats of *O. bonnemaisonii* were observed in the Rairua bay, in 2010 together with very small mats of *H. glutinosum, Anabaena* sp., and *Leptolyngbya* sp.

3.2.3.2 "Motu De La Femme" Area

Near the "Motu de la femme", *O. bonnemaisonii* was found in April 2007, covering *Halimeda* sp. in the channel between the "motu" and the main island at the 1 to 5 m depth. In this area, the local population reported on past excavations of coral slush to construct the road. Strong currents through this channel have been noticed during the spring tides. In the course of our four following sampling campaigns, no blooms of *Oscillatoria* were observed. However, with the exception of February 2010 mission which took place right after the tropical hurricane Oli, which removed most of the benthic cover, extensive mats of *Leptolyngbya* sp. have developed in this area.

3.2.3.3. "Motu Piscine" Area

In February 2010, near the motu "Piscine", some coral pinnacles were covered by mats of *H. lyngbyaceum* and *H. coccineum*.

3.3. Toxicological Analysis of Lipid-Soluble Extracts

The overall results from experiments aiming to assess the toxicity of the lipid-soluble extracts are summarized in Table 1.

3.3.1. MBA

Samples of cyanobacteria and giant clams from Lifou and Emao could not be analyzed for MBA because of insufficient biomass. Only samples collected in Raivavae were tested using this method.

a - Cyanobacteria

Lipid-soluble extracts of the cyanobacterium *Oscillatoria bonnemaisonii* collected in April 2007 in the bay of Rairua (Raivavae) showed toxicity in mice < 0.2 $MU.mg^{-1}$ of extract and those of samples collected in the bloom of "Motu de la femme" exhibited a toxicity estimated at < 0.5 $MU.mg^{-1}$ of extract.

b - Giant Clams

Lipid-soluble extracts of giant clams collected in the bay in front of Rairua provoked toxicity in mice, estimated at 0.6 $MU.g^{-1}$ of flesh.

For the other locations, the toxicity was estimated at <0.4 MU. However, for both cyanobacteria and giant clam toxic extracts, the observed symptoms were not characteristic of those usually provoked by CTXs, as neither diarrhea nor cyanosis was recorded and the intensity of symptoms was not proportional to the injected doses.

To detect the presence of VSSC activator toxins such as CTXs, lipid-soluble extracts of cyanobacteria, giant clams were subsequently assayed in neuroblastoma cytotoxicity assay (NCA) and in receptor binding assay (RBA) (Table 1). As for the fish, the toxicity was evaluated only with RBA potency.

3.3.2. NCA

a - Cyanobacteria

Using NCA, samples from Lifou showed a higher toxicity without ouabaïn/veratridine treatment (OV-) than with that treatment (OV+). The highest cytotoxicity was observed with *P. laysanense* and the weakest with *H. glutinosum*.

Conversely, in the three samples of Emao, *H. glutinosum*, *Anabaena* sp. and *Symploca hydnoides*, provoked a higher toxicity with OV+ than without it (OV-). This difference is more obvious in the fraction F3, where they appeared non-toxic in OV- and toxic in OV+ with EC_{50} values around 20 $\mu g.mL^{-1}$ of the extract.

Table 1. Toxicity data obtained via the neuroblastoma cytotoxicity assay (NCA) and the receptor binding assay (RBA) performed on lipid-soluble extracts of cyanobacteria and giant clams

Seafood	Dates	Locates	NCA				RBA	
			without OV		with OV			
			F 2	F 3	F 2	F 3	F 2	F 3
			µg.mL^{-1}				ng P-CTX-3C eqv.mg^{-1}	
Cyanobacteria	Dates	Locates	NCA				RBA	
H. lyngbyaceum [(a)]	2005/03	Lifou (Hunëtë, toxic area)[(a)]	138	NCA-	197	NCA-	2.41	-
H. lyngbyaceum, Oscillatoria sp., P. laysanense	2007/02	Lifou (Hunëtë, toxic area)	114	NCA-	260	NCA-	2.84	-
H. glutinosum	2008/02	Lifou (Hunëtë, toxic area)	320	NCA-	NCA-	NCA-	RBA-	-
S. weissii	2008/06	Lifou (Hunëtë, toxic area)	125	NCA-	NCA-	NCA-	0.86	-
O. cf bonnemaisonii	2009/02	Lifou (Hunëtë, toxic area)	142	NCA-	254	NCA-	1.03	-
P. laysanense	2009/11	Lifou (Hunëtë, toxic area)	36	NCA-	97	NCA-	1.1	-
H. glutinosum / Anabaena	2009/10	Emao (Lausake)	99 /NCA-	NCA- /NCA-	75	25	-	-
Anabaena sp.	2009/10	Emao (Lausake)	173 /26	NCA-	57	21	-	-

Table 1. (Continued)

Seafood	Dates	Locates	NCA				RBA	
Symploca hydnoides	**2009/10**	**Emao (Lausake)**	**105**	**NCA-**	**96**	**20**	**-**	**-**
O. bonnemaisonii	**2007/04**	**Raivavae (Rairua)**	**-**	**-**	**-**	**-**	**2.32**	
O. bonnemaisonii	**2007/04**	**Raivavae (Motu de la femme)**	**-**	**-**	**-**	**-**	**3.9**	
O. bonnemaisonii	**2007/09**	**Raivavae (Rairua)**	**137**	**69**	**NCA-**	**NCA-**	**9.4 ± 4.56 (n=12)**	
O. bonnemaisonii	**2008/05**	**Raivavae (Rairua)**	**34**	**NCA-**	**6**	**10**	**1.04**	
O. bonnemaisonii	**2009/02**	**Raivavae (Rairua)**	**58**	**NCA-**	**128**	**NCA-**	**3.6**	**0.7**
O. bonnemaisonii	**2009/02**	**Raivavae (Rairua)**	**NCA-**	**NCA-**	**NCA-**	**NCA-**	**3.49**	
O. bonnemaisonii	**2009/02**	**Raivavae (Rairua)**	**NCA-**	**NCA-**	**NCA-**	**NCA-**	**1.49**	
O. bonnemaisonii	**2009/02**	**Raivavae (Rairua)**	**NCA-**	**NCA-**	**NCA-**	**NCA-**	**1.51**	
H. lyngbyaceum	**2010/02**	**Raivavae (Motu Piscine)**	**-**	**-**	**-**	**-**	**9.96**	**3.79**

Bénitiers	Dates	Locates	NCA				RBA	
	2005/03	Lifou (Hunëtë)	-	-	-	-	7.95	-
Seafood	Dates	Locates	NCA				RBA	
	2005/11	Lifou (Hunëtë)	-	-	-	-	15.65	-
	2007/02	Lifou (Hunëtë)	132	-	123	-	-	-
	2008/02	Lifou (Hunëtë)	as OV+	NCA-	40 - 215	NCA-	-	-
	2009/02	Lifou (Hunëtë)	as OV+	NCA-	6 - 281	NCA-	-	-
	2009/10	Emao (Lausake)	NCA-/57	NCA-	18	40	-	-
	2009/10	Emao (Waina)	NCA-	NCA-	NCA-	NCA-	-	-
	2007/04	Raivavae (Rairua)	NCA-	NCA-	16	NCA-	10.8	
	2007/09	Raivavae (Rairua)	71	NCA-	25	1	12.1	
	2008/05	Raivavae (Rairua)	NCA-	NCA-	NCA-	1.6	8.1	
	2009/02	Raivavae (Rairua)	NCA-	NCA-	1	NCA-	2.6	RBA-
	2010/02	Raivavae (Rairua)	-	-	-	-	6.61	
	2007/04	Raivavae (Motu femme)	NCA-	69	42	59	64.2/52.8	

Table 1. (Continued)

Seafood	Dates	Locates	NCA				RBA	
	2008/05	Raivavae (Motu femme)	NCA-	NCA-	0.9	0.9	7.2	
	2009/02	Raivavae (Motu femme)	NCA-	NCA-	NCA-	NCA-	2.2	
	2010/02	Raivavae (Motu femme)	-	-	-	-	4.79	
	2007/04	Raivavae (Motu Mano)	NCA-	NCA-	NCA-	NCA-	RBA-	
	2007/09	Raivavae (Motu Mano)	NCA-	NCA-	NCA-	NCA-	RBA-	
	2009/02	Raivavae (Motu Mano)	NCA-	NCA-	NCA-	NCA-	3.7	RBA-
	2010/02	Raivavae (Motu Mano)	-	-	-	-	1.2-	
	2007/09	Raivavae (Motu Piscine)	-	-	-	-	RBA-	
	2008/05	Raivavae (Motu Piscine)	NCA-	NCA-	NCA-	NCA-	6.5	
	2009/02	Raivavae (Motu Piscine)	NCA-	NCA-	NCA-	NCA-	RBA-	
	2010/02	Raivavae (Motu Piscine)	-	-	-	-	4.19/2.41	

[a] Laurent *et al*., 2008.

The mention "RBA-" was applied for the values < 0.86 ng P-CTX-3C eqv.g^{-1} of flesh.

The mention "NCA-" was applied for R^2 values < 0.9 or for matrix effect.

In Raivavae, only *O. bonnemaisonii* samples collected in front of Rairua in September 2007, May 2008 and February 2009 were analyzed with NCA. With the sample of September 2007, the cytotoxic effect was visible only in OV- and more particularly in the fraction F3. Conversely, the sample of May 2008 showed strong cytotoxicity with an EC_{50} of 6.38µg.mL^{-1} in OV+ comparatively to 33.87 µg.mL^{-1} in OV-. In the same manner, the fraction F3 in OV+ was cytotoxic (10.09 µg.mL^{-1}) while it was non-toxic in OV-. In February 2009, four samples were collected and only one manifested a weak cytotoxicity in F2 fraction but nevertheless more obvious with OV- than with OV+ (Figure 8).

b - Giant Clams

The giant clams of Lifou were analyzed only for their F2 fractions. They provoked a cytotoxicity showing with EC_{50} values varying from 6 to 281 µg.mL^{-1}, which do not seem to be dependent of the OV treatment. No difference was observed between giant clams from the presumed toxic area and the safe area.

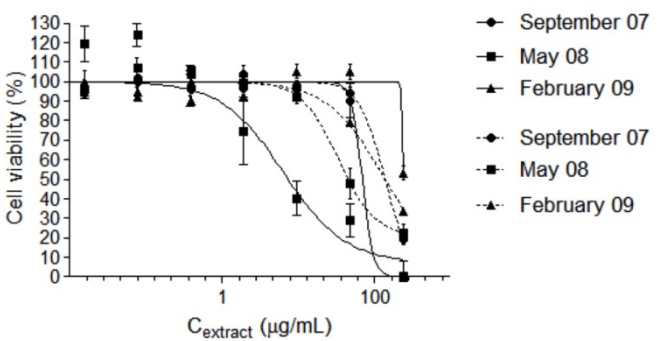

Figure 8. Effect on the viability of the Neuro-2a cells with (full lines) or without (doted lines) OV treatment of *Oscillatoria bonnemaisonii* lipid-soluble extracts from Rairua bay collected at different saisons (September 2007, May 2008 and February 2009).

In Emao, a difference of toxicity was obvious between samples collected in front of Lausake and those collected in front of Wiana. Samples from Lausake caused a cytotoxic effect depending on the treatment (OV-dependent) and lightly greater in the F2 fraction than in F3 one, while samples from Wiana were showed as non-toxic.

In Raivavae, a good correlation was observed between the giant clam cytotoxicity and the presence of cyanobacterial blooms. This cytotoxicity was dependent on the presence of OV, with the highest level observed either in fraction F2 or F3. Concerning more precisely the Rairua bay, the EC_{50} values of F2 fractions in OV+ varied from 5.9 $\mu g.mL^{-1}$in April 2007 to turn, unexpectedly, non-toxic in May 2008. But, in this period, EC_{50} value of F3 fraction showed a strong toxicity (1.59 $\mu g.mL^{-1}$).

The toxicity of giant clams collected near "Motu de la femme" seems to lessen over time. In April 2007, during the large bloom of *O. bonnemaisonii*, the observed EC_{50} values were in OV+ : 41.8 $\mu g.mL^{-1}$in F2 and 58.7 $\mu g.mL^{-1}$in F3; then, in May 2008, they were 0.9 $\mu g.mL^{-1}$in F2 and in F3; while in February 2009, the toxins were not detectable.

In "Motu Mano", no cytotoxicity was observed in the samples collected in April 2007, September 2007 or February 2009; in fact no more than in the extracts of giant clams collected in May 2008 and February 2009 in "Motu Piscine" that served as a healthy control.

c - Fish

Fish toxicity in Lifou was estimated by NCA for 22 parrotfishes (*Scarus schlegeli* and *S. rivulatus*) caught in February 2007, 2008 and 2009. The percentage of toxic fishes likely to poison human (> 540 µg eqv extract.mL^{-1} in OV+) was about 60% in 2007, 85% in 2008 and at the most 50% in 2009.

Fish toxicity in Emao and Raivavae was analyzed by RBA (see below).

3.3.3. RBA

P-CTX-3C was used as an internal standard to estimate the toxicity values monitored in both cyanobacteria and giant clam samples. Based on RBA results, both cyanobacterial and giant clam samples were classified into two groups as follows:

- Samples that failed to compete with [^{3}H]PbTx-3 (RBA^-) or those with RBA_{cyanos}< 0.34 ng P-CTX-3C eqv.mg^{-1} or RBA_{clams}< 0.86 ng P-CTX-3C eqv.g^{-1} were regarded as non toxic samples

- Samples with $RBA_{cyanos} \geq 0.34$ ng P-CTX-3C eqv.mg^{-1} extract or $RBA_{clams} \geq 0.86$ ng P-CTX-3C eqv.g^{-1} flesh were those able to compete with [^{3}H]PbTx-3 (RBA^+), and were thus regarded as toxic samples.

a - Cyanobacteria

RBA values of cyanobacteria in Lifou are species-dependent. The bloom of *H. glutinosum* was the only one that was found to be non-toxic (RBA^-). Increasing RBA^+ values were found in blooms of *S. weissii*, *O. bonnemaisonii*, *P. laysanense* and *H. lyngbyaceum*. The multispecies bloom of *H. lyngbyaceum*/*Oscillatoria* sp. showed the strongest toxicity with 2.84 ng P-CTX-3C eqv.mg^{-1} of extract.

The cyanobacterial blooms from Emao were analyzed with NCA but not with RBA (see above).

In Raivavae, RBA values of blooms of *O. bonnemaisonii* collected in Rairua bay showed that the toxicity can be ten times higher from one season to another. For example, when the bloom was very patchy as in the cold season in September 2007, the RBA value was 9.4 ± 4.6 ng P-CTX-3C eqv.mg^{-1} of extract, but decreased for an order of magnitude in May 2008 with 0.94ng P-CTX-3C eqv.mg^{-1} of extract. Intermediate values were observed in April 2007 and in February 2009 (Figure 9). The same species collected in "Motu de la femme" showed RBA values of 3.9 ± 2.2,in April 2007.

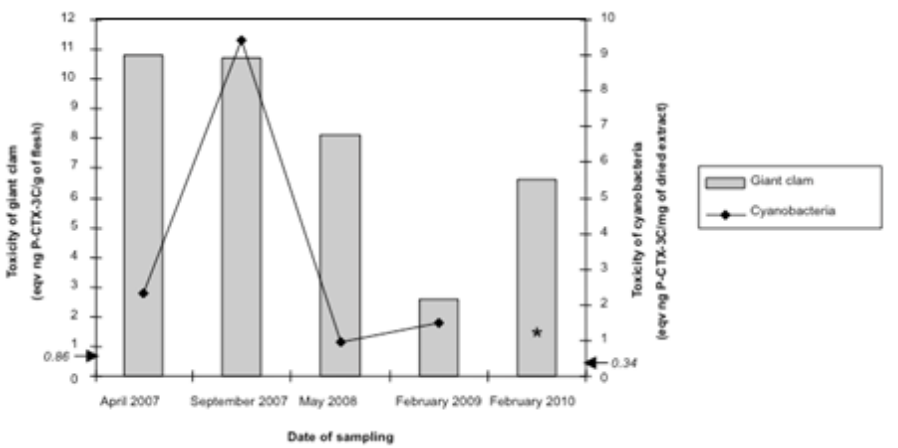

Figure 9. RBA toxicity of cyanobacteria and giant clams collected in Rairua area (Raivavae island, French Polynesia). The asterix shows the absence of cyanobacteria. Bold arrows show the limits of RBA : values obtained above this limit are for samples

that were able to compete with tritiated brevetoxin (RBA+), but below this threshold, samples are considered negative (RBA-).

H. lyngbyaceum bloom was observed in February 2010 in "Motu Piscine", an area where population used to offer tourists sample giant clams for tasting. Its toxicity was estimated to be 9.96 ng P-CTX-3C eqv.mg^{-1} of F2 extract and 3.79 ng P-CTX-3C eqv.mg^{-1} of F3 extract.

b - Giant Clams

In Lifou, CTX concentration in giant clams collected in March 2005 close to the *H. lyngbyaceum* bloom was estimated to be 7.95 ± 1.06 ng P-CTX-3C eqv.mg^{-1} of extract (Laurent *et al.,* 2008), whereas it was twice that value eight months later.

The RBA values of the giant clams collected from the Rairua bay, in the "high-risk area" designated by the Raivavae's inhabitants, varied according to season. Successively, RBA values were 10.8 (April 2007), 10.73 ± 1.92 (Sept. 2007), 8.1 (May 2008), 2.6 (Febr. 2009) and 6.61 (Febr. 2010) as expresses in ng P-CTX-3C eqv.g^{-1} of flesh (Figure 9). The giant clams from "Motu de la femme", where we observed also a large bloom of *O. bonnemaisonii* in April 2007, showed RBA value of 58.5 ng P-CTX-3C eqv.g^{-1} of flesh at this period, while these values were ten to twenty times weaker in May 2008, February 2009 and February 2010, during those times the blooms did not occur. In "Motu Piscine", giant clams were non-toxic in September 2007 and February 2009, but showed a toxicity on two coral pinnacles, where at the time a bloom of *H. lyngbyaceum* was observed in flesh of 6.5 ng P-CTX-3C eqv.g^{-1} in May 2008, and 2.41 and 4.19 ng P-CTX-3C eqv.g^{-1} in February 2010.

In waters surrounding "Motu Mano", where giant clams are currently exploited, specimens were found to be RBA- in April and September 2007 and slightly RBA+ in February 2009 and 2010.

c - Fish

In Emao, 53 fishes were collected with 43 specimens belonging to 4 species of herbivores. 32 specimens were caught in Lausake and 11 in Wiana. Of the 10 carnivorous fishes that belonged to 7 species, only three specimens were caught in Wiana.

RBA values were not correlated with size or weightof the fish as observed earlier by Darius *et al.*(2007)and Chinain *et al.*(2010). Regarding RBA results, only two specimens belonging to the species *Chlorurus sordidus* and *Lethrinus harak* from Wiana were considered non toxic (<0.31 ng P-CTX-3C eqv.g^{-1} of

flesh). Overall, levels of toxicity observed in Wiana were approximately two times lower than in Lausake, as illustrated by specimens of the parrotfish *C. sordidus* and thesurgeon fish *Ctenochaetus striatus* (Table 2).

In Raivavae lagoon, among 160 specimens collected from various sampling areas and belonging to the species most often cited by the population as either ciguatera non-toxic or edible, only 36 % could be considered as safe (<0.31 ng P-CTX-3C eqv.g^{-1} of flesh) (Chinain *et al*., 2010). The highest RBA values were 5.58 and 4.67 ng P-CTX-3C eqv.g^{-1} of flesh, which respectively reported in a surgeonfish, *Scarus altipinnis* and in a unicornfish, *Naso unicornis,* respectively.

Although *N. unicornis* and *S. altipinnis* were found toxic in all stations around the island, the area bounded as high ciguatera risk by the population correlated well with the assessment based on toxicological assays.

Table 2. Comparative toxicity observed in RBA of two species of herbivorous fishes (*Chlorurus sordidus* and *Ctenochaetus striatus*) according to their collecting site

Fish species		Lausake	Wiana
Chlorurus sordidus	Number of fish	8	3
	RBA*	2.1 ± 1.3	0.8 ± 0.4
	Size**	22.8 ± 1.9	21.0 ± 4.1
	Weight***	233.9 ± 57.9	213.7 ± 85.0
Ctenochaetus striatus	Number of fish	20	8
	RBA	4.8 ± 2.0	2.1 ± 1.0
	Size	16.3 ± 3.0	16.6 ± 2.5
	Weight	110.0 ± 53.4	123.0 ± 44.4

*RBA : mean ± standard deviation expressed in ng P-CTX-3C eqv.g^{-1} of flesh.
**Size : mean ± standard deviation expressed in cm.
***Weight : mean ± standard deviation expressed in g.

3.4. Toxicological Analysis of Water-Soluble Extracts

Symptoms exhibited by mice injected intraperitoneally with water-soluble extracts or acid-methanol extracts of either cyanobacteria or giant clams from

Lifou included ataxia, laboured breathing, frequently convulsive spasms, and paralysis followed by death occurring within the first minutes with doses up to 4-5 $mg.g^{-1}$ body weight. Whereas a complete recovery was observed at doses < 4 $mg.g^{-1}$ body weight.

4. DISCUSSION

Seafood poisonings can result from ingestion of different marine toxic products contained in finfish or invertebrates. These poisonings are not always due to a single toxin or a particular group of toxins, and they do not necessarily originate from a single microorganism. Otherwise stated, complex mixtures of toxins occurring in mixed blooms would be the starting point of a poisoning event. Therefore to analyze the toxicity of the seafood and determine the origins of marine biotoxins, the applied bioassays require high specificity and sensitivity.

In the present case, we face a new type of poison that involves new and less known toxins producing microorganisms as well as other vectors than those implicated in CFP. Accordingly, we need to analyze the toxicity of our samples by applying method that includes toxins other than CTXs. For this reason, we used three different bioassays that are distinguished by their specificity (MBA, NCA and RBA).

Until very recently, MBA was the official method of detection and determination of toxin content in seafood products. The mouse bioassay entails injection of extract from suspected seafood into 2-3 mice per sample and monitoring their time of death. The mouse bioassay reveals the overall toxicity, but has a low sensitivity and does not identify of the involved toxin(s) qualitatively. In November 2010, the European Community has officially approved LC-MS/MS replacement for lipophilic toxins MBA (http://ec.europa.eu/dgs/health_consumer/index_en.htm).Determination of the most appropriated alternative method to animal bioassays for the detection of a large range of marine toxins is of paramount importance and is the subject of much of the current work (Cañete and Diogene, 2008; 2010; Botana *et al*., 2009; 2010; Caillaud *et al.,* 2010, Campbell*et al*., 2011).

NCA represents a more sensitive and specific alternative to the Mouse Bioassay, which also reduces the ethical implications of animal use (Ledreux *et al*., 2009; Caillaud *et al*., 2009and 2010). Its specificity mainly concerned the detection of cytotoxins that act on sodium channels. This functional restriction may give confusing results in the presence of toxin mixtures. The

use of ouabain/veratridine at different concentrations, which enhance sodium entry into the cells, allows the detection of different toxins according to their action on sodium channels i) activators as ciguatoxins and brevetoxins or ii) inhibitors as saxitoxins and tetrodotoxins (Ledreux *et al.,* 2009). In our studies, the concentration of ouabain/veratridine was established to obtain a weak cytotoxicity of 20%, which allows the observation of cytotoxicity due to ciguatoxins or similar toxins acting on VSSC. Without the treatment with ouabain/veratridine, the cytotoxic activity of CTXs could not be observed.

RBA is more specific. This assay utilizes radio labelled toxin standard in competition with toxins in the sample for the molecular site of action. The results of this assay correlate well with the potential toxicity of the sample, providing that the receptor used is the particular biological target where the toxins exert their effect. In our case, tritiated brevetoxin was used (Dechraoui *et al*., 2009; Bottein Dechraoui *et al.,* 2005). Brevetoxin is a commercialized toxin produced by the dinoflagellate *Karenia brevis*, which acts on the same site of action as CTXs, *i.e.* at the site 5 of VSSC, but with a lower affinity (Poli *et al.,* 1986).

Due to the complex matrix of samples for both NCA and RBA, an additional chemical purification step is necessary to avoid artefacts or matrix effect. A C_{18} Sep-Pak® cartridge following a liquid-liquid partition is used to give three fractions of different polarity. The intermediate F2 fraction contains usually all the known CTXs ranging from the least polar isolated from dinoflagellates, to the most polar extractedfrom carnivorous fishes. The fraction F1 would contain the more polar substances, while the fraction F3 would regroup toxic compounds, *i.e.* the less polar products than CTXs.

No effect was observed in NCA and RBA with the F1 fractions of cyanobacteria and giant clams. In contrast, some cytotoxic effects were observed with the F3 fraction of cyanobacteria and giant clams from Raivavae and Emao, showing the presence of less polar toxins than CTXs.

The results of the toxic analysis, by NCA or RBA, may differ depending on the cyanobacterial specific support, the appropriateness of the sampling method or on the purity of the obtained sample. For example, *Oscillatoria* samples taken from *Halimeda* meadows by an under-water suction device were more uniform. It was much more difficult to obtain clean samples by scraping *H. lyngbyaceum* from their dead coral support.

In September 2007, 12 samples of cyanobacteria were collected from different stations taken from within the Rairua bay (Raivavae). Their RBA values were found to vary from 3.05 to 19.14 ng P-CTX-3C eqv.mg^{-1} of extract, suggesting variability in toxin production from one local population to

another. This observation was also made in the same area of the Rairua bay in February 2009, where the RBA values of four samples varied from 1.49 to 3.6 ng P-CTX-3C eqv.mg^{-1} of extract. As for the toxin production in dinoflagellates, the toxinogenic potential is strain-dependent.

Concerning the giant clam extracts from Emao and Raivavae, the cytotoxicity on neuroblastoma cells was mainly found in the samples collected in the sites where Oscillatoriales mats were observed, that is to say in front of Lausake for Emao and in front of Rairua and near the "Motu de la femme" for Raivavae. Such a correlation is also significant in RBA for giant clams from Raivavae. These results fully corroborate the designation of a "high-risk area" by the local population of both islands. In Lifou, the size of the areas, toxic or safe, is very small (300 meters) and the risk differentiation seems to be less obvious.

In the three islands, giant clams seem to accumulate elevated doses of toxins acting on the site 5 of VSSC (until 60 ng P-CTX-3C eqv.g^{-1} of flesh for the samples collected in the "Motu de la femme" during the large *O. bonnemaisonii* bloom in April 2007), higher than the safety limit of 0.86 ng P-CTX-3C eqv.g^{-1} of flesh above which a giant clam is considered potentially dangerous.

The lack of close correlation between the NCA and RBA results shows the complexity of this toxicological problem. We can hypothesize that cyanobacteria would have a complex toxinogenic potential with, i) paralyzing water-soluble toxins (observed by MBA), ii) lipid-soluble cytotoxins acting not-specifically on sodium channels (cytotoxicity observed by NCA in OV- condition), iii) cytotoxins specifically acting on sodium channels (cytotoxicity observed by NCA only in OV+), and iv) toxins acting on site 5 of sodium channels like the CTXs (observed by RBA). Depending on the toxin profile of cyanobacteria or giant clams, the different toxins acting in synergy or by antagonism on the channels, the results of the bioassays could be biased and the action of one toxin could be hidden by the action of another one.

The exact nature of the lipid-soluble toxins acting specifically or not on VSSC is still unknown. The toxins, which enhance sodium channels, could be antillatoxin, brevetoxin, CTX or other polyethers. However, antillatoxin seems to be a too small molecule to be implicated since chemical and RBA analysis performed with giant clam extracts of Lifou showed that these toxins had a polarity close to that of P-CTX-3C and a MW of about 1,000 Da (Laurent *et al.,* 2008). The presence of brevetoxin cannot be completely dismissed, as well, since the temperature-related dysesthesia felt by patients of Lifou, Emao and Raivavae, has also been reported in Neurotoxic Shellfish Poisoning, which

is caused by human consumption of shellfish contaminated by these toxins (Friedman *et al.,* 2008). However, brevetoxins are known to be often associated with large fish mortalities (Magana *et al*., 2003) and to be produced by a red tide dinoflagellate (*K. brevis*), which were never reported in the three locations.

Ehrenreich *et al.* (2005) had previously identified in diverse marine and freshwater cyanobacterial cultures, the sequence of gene fragments from nonribosomal peptide synthetases and modular polyketide synthases, this last molecular system being implicated in polyether compound synthesis that could include the structural building block of CTXs.

Therefore, our results are more in favour of the presence of CTX-like compounds in our cyanobacterial extracts, all the more since RBA is a test specifically designed to detect the presence of toxic compounds such as brevetoxins and CTXs.

Regarding the toxicity of water-soluble extracts, we failed in confirming the presence of anatoxin-a in Oscillatoriales samples - as previously demonstrated in *Hydrocoleum* (Mejean *et al.,* 2010) - and in giant clams from Emao or Raivavae. However, cases of dog mortality following the consumption of giant clams in two Polynesian atolls (Fangatau and Pukarua) and also in Bora Bora in 1964 (Bagnis, 1967) ressembled the illness and death of dogs by severe paralysis (notably respiratory muscles) conclusively associated with anatoxin-a-producing freshwater cyanobacteria *Oscillatoria* spp. (Edwards *et al.,* 1992) and *Phormidium favosum* (Gugger *et al.,* 2005). Later, Cadel-Six *et al.*(2007) showed that different genotypes of anatoxin-producing freshwater cyanobacteria coexist in the Tarn River, France. This neurotoxin, which has never been detected in marine cyanobacteria, nor implicated in human poisonings, has to be considered as a potential health hazard for seafood consumers.

In Raivavae, as in Lifou, our findings suggest a relationship between anthropogenic disturbances of the reef ecosystem, the development of Oscillatorialean cyanobacterial blooms and the resulting high CFP-like incidents following the consumption of giant clams from contaminated areas. Indeed, giant clams collected in the immediate vicinity of cyanobacterial blooms were found to be toxic, likely due to the presence of lipid-soluble toxins and water-soluble paralysing toxins. In Emao, the anthropogenic disturbance is not obvious even though the reef is much more deteriorated in front of Lausake than in front of Wiana, the neighboring village.

Therefore we postulate that these different toxins may accumulate in filter-feeding organisms and their predators during cyanobacterial blooms.

However we are aware that the study of the toxinogenesis of axenic cyanobacterial culture will help confirm or refute this hypothesis, eliminating the possibility of toxicity due to another less common microorganisms.

Although only some cases of giant clam poisonings could be documented in Lifou, Emao and Raivavae through population interviews, it is likely that the prevalence of this type of poisoning is much higher than it was reported. One reason for this is the ignorance of the physicians and other healthcare providers, who would continue to incriminate previous or simultaneous fish consumption. Giant clams are not the only shellfish involved in this kind of intoxication. Inhabitants of Emao specified also another bivalves (e.g. *Atactodea striata*) and gastropods (e.g. *Nerita polita* and *Trochus* sp.) as being poisonous.

In Lifou, for more than a half of the cases, the implicated fishes were herbivorous as unicornfish (*Naso* sp.), or grazers as parrotfish (*Scarus* sp.), or molluscivorous as the long-nosed emperor fish (*Lethrinus* sp.). In Emao, the three fish families known as the most dangerous by the communities are the *Acanthuridae* (*Ctenochaetus striatus* in the first place), the *Scaridae* (mainly *Chlorurus sordidus*) and the *Lethrinidae* (*Lethrinus harak*). In Raivavae, the toxicity monitoring in various fish species belonging to the early stages of the ciguatera food chain, *e.g.* unicornfish ("ume", local name for *N. unicornis*) and 2 species of parrotfishes ("roro" and "haumeretue", local names for *Scarus altipinis*), revealed that these species were particularly toxic, including in areas deemed "healthy" by the population. These toxicity data were also consistent with the preliminary results from the analysis of epidemiological medical records compiled by the Raivavae Medical Centre since April 2007 (Chinain *et al.,* 2010).

Taken together, these observations strongly suggest that cyanobacterial toxins may enter the food chain directly via grazer fishes which graze on cyanobacterial mats, or via molluscivorous fishes which prey on contaminated molluscs; this original pathway would account for the strong involvement of these fish families in Lifou, in Emao, and in Raivavae intoxications.

The overall results are in favour of a new trophic pathway of human poisoning via molluscs that could be referred to as "Ciguatera Shellfish Poisoning" (Figure 10). Indeed, the symptoms experienced by the patients include the *pathognomonicreversal*of *temperature sensation; therefore, the term "ciguatera" can still apply to this new form of intoxication*. It is also noteworthy that the term "cigua" is the Cuban name for a small turbinid shell *Livona pica,* which produces a similar illness upon ingestion (Poey, 1866).

Pelagic Oscillatorialean cyanobacteria, morphologically and genetically very close to the genus *Hydrocoleum* (Abed et *al.,* 2006), may be involved in such phenomenon, as a similar toxicity has been observed in *T. erythraeum* (Kerbrat *et al*., 2010) unlikely to be contaminated by microalgal (*i.e.* dinoflagellates) toxins.

These data provide a better understanding of the ecotoxicological environmental phenomenon of CFP, and would hopefully contribute to the improvement of seafood poisoning risk assessment and management programs in coral reef regions, that are so far limited to the monitoring of toxigenic dinoflagellates. Indeed, our results strongly suggest that the presenceof potentially toxic cyanobacteria in high risk areas should also be taken into account, all the more so, since these microorganisms are likely to proliferate in the context of global warming (Chateau-Degat *et al*., 2005).

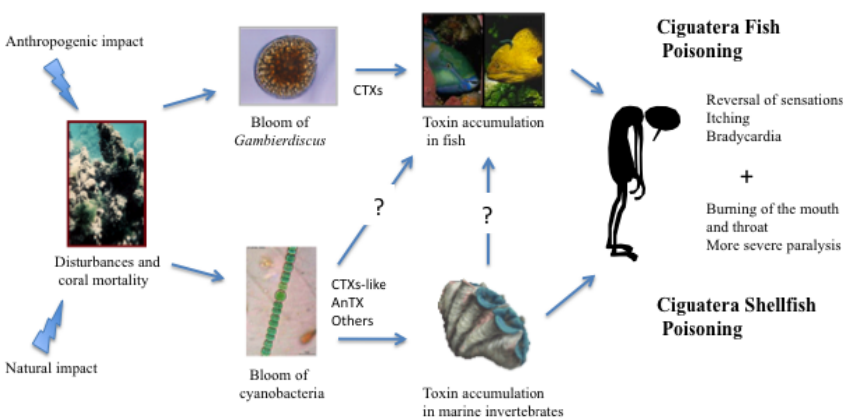

Figure 10. Ciguatera Shellfish Poisoning: a new trophic pathway of human poisoning via marine mollusks.

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