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Detection of ciguatoxin-like and paralysing toxins in *Trichodesmium* spp. from New Caledonia lagoon

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ABSTRACT

Marine pelagic cyanobacteria *Trichodesmium* are widespread in the New Caledonia lagoon. Blooms of these Oscillatoriales are suspected to be a potential source of toxins in the ciguatera food chain and were previously reported to contain certain types of paralysing toxins. In the present study, toxicity experiments were conducted on lipid- and water-soluble extracts of freeze-dried samples of these cyanobacteria. Lipid-soluble fractions revealed a ciguatoxin-like activity in both *in vivo* (mouse bioassay) and *in vitro* (mouse neuroblastoma cells assay and receptor binding assay using tritiated brevetoxin-3) assays. The water-soluble fractions tested on mice exhibited neurotoxicity with paralytic symptoms. These toxicities have also been observed with benthic filamentous cyanobacteria within the Oscillatoriales order, also collected in New Caledonia. This study provides an unprecedented evidence of the toxicity of *Trichodesmium* species from the New Caledonia lagoon. This survey also demonstrates the possible role of these cyanobacteria in ciguatera fish poisoning.

1. Introduction

The genus *Trichodesmium* (Oscillatoriales) consists of filamentous cyanobacterial species. An important component of the phytoplankton community observed during the bloom in tropical and subtropical seas, *Trichodesmium* spp. is widespread in oligotrophic waters and is a critical primary producer. These microorganisms contribute greatly to new nitrogen influx (diazotrophy) and thus to global cycling of nutritional elements (Capone et al., 1997; Carpenter et al., 2004). This genus, both freshwater and marine, is composed of five species, namely *Trichodesmium erythraeum*, *T. thiebautii, T. hildebrantii*, *T. contortum*, and *T. tenue* (Carpenter et al., 1993; Janson et al., 1995, 1999). *T. erythraeum* and *T. thiebautii* are most prevalent in New Caledonia waters (Rodier and Le Borgne, 2010). Trichomes of these non-heterocystous pelagic cyanobacteria group mix to form colonies, varying from red to yellow in color depending on the age of the bloom and the concentration of the species.

Trichodesmium blooms are easily visible as they accumulate on sea surface. In New Caledonia waters, *Trichodesmium* blooms generally occur during the austral summer (September to March). Their patchy spatial and temporal distribution is usually connected to the physico-chemical variability of the water masses (Tenório et al., 2005; Rodier and Le Borgne, 2008, 2010). As a matter of fact, bloom occurrences are positively correlated with temperature, salinity and nutrients regime, while negatively correlated with the wind speed, rainfall, sea level pressure and dissolved oxygen (Chang et al., 2000; Thajuddin and Subramanian, 2005; Bhat et al., 2006; Rodier and Le Borgne, 2008).

Despite the number of surveys done on the ecological aspect of *Trichodesmium* spp. and their importance to the coral reef ecosystems, their toxicity remains sparsely documented (Sellner, 1997). The stochastic nature of the blooms and the difficulties inherent in establishing laboratory cultures have hampered such toxicological studies (Chen et al., 1996; Bell et al., 2005).

In 1991, Hawser reported the death of oysters following *Trichodesmium* blooms (Hawser et al., 1991). The cyanobacteria had been collected and tested on various species of zooplankton. *Trichodesmium* demonstrated mortality on certain crustaceans (brine shrimp and two species of copepods); although grazers that are known to feed on *Trichodesmium* (*Macrosetella gracilis* and *Miracia efferata*) were not affected. However, no information was provided on the nature of toxins involved (Hawser et al., 1992; O'Neil and Roman, 1992).

Based on chemical analysis studies, Hahn and Capra (1992) were the first to imply that *T. erythraeum* could be a potential

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source of toxin in Ciguatera Fish Poisoning (CFP), a typical food-borne intoxication in the tropics due to the ingestion of fish contaminated with lipid-soluble ciguatoxins (CTXs) (Lehane and Lewis, 2000; Nicholson and Lewis, 2006; Friedman et al., 2008). The compounds extracted from *T. erythraeum* and from molluscs samples collected during and shortly after this cyanobacterium bloom were positive for CTXs-like toxins (Hahn and Capra, 1992).

Shortly after, Endean et al. (1993) demonstrated that the toxin profiles of the lipid-soluble and also water-soluble extracts from *T. erythraeum* were impossible to differentiate from the corresponding fractions obtained from the flesh of the pelagic carnivore *Scomberomorus commersoni* which are often implicated in CFP intoxication. Chromatographic elutions of water-soluble and lipid-soluble fractions from both samples showed an alkaloid in addition to a peptide and CTXs-like compounds, respectively. This indicated that *Trichodesmium* spp. could be the source of toxins carried by some ciguateric fish and, that the toxins released by these cyanobacteria may contribute to CFP intoxication.

It is not yet known whether New Caledonia strains of *Trichodesmium* spp. produce neurotoxins similar to those reported in the above studies (Hahn and Capra, 1992; Endean et al., 1993). Based on the biological activities of the pelagic bloom samples of *T. erythraeum* and *T. thiebautii* which occurred from January 2007 to February 2008 in the lagoon of New Caledonia, the present study intends to address this particular issue. These samples were treated using extractive methods suitable to separate lipid-soluble compounds (e.g. CTXs) and water-soluble compounds (e.g. Paralytic Shellfish toxins (PSTs) or anatoxin-a and its homologues). For comparison, similar preliminary analyses were also carried out on certain marine benthic Oscillatoriales (*Hydrocoleum*, *Oscillatoria* and *Phormidium*) collected in a human impacted area offshore of Lifou (New Caledonia) where numerous CFP cases were reported (Laurent et al., 2008).

2. Materials and methods

All reagents and chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise stated. Solvents were of analytical grades and were purchased from Prolabo (Paris, France).

2.1. Sampling sites and cyanobacteria collection

Trichodesmium samplings took place primarily during the hot season, in the southern lagoon of New Caledonia, as soon as blooms were reported (Table 1). Samples were collected on the sub-surface (0–1 m) and concentrated with a 35 μm phytoplankton net. All samples were delicately handled to avoid cell lysis and toxins release. The trichomes were washed thoroughly in order to discard macroalgae, phanerogams and other organic or inorganic debris and further concentrated by reversing the sampling bottles. The buoyancy property conferred by intracellular gas vacuoles separates the cyanobacteria from other organisms in layers in the water column. Specimens from each batch were fixed in 5% formaldehyde solution for morphological identification purposes and the concentrated samples were frozen and freeze-dried until further studies.

Benthic Oscillatoriales were collected on three occasions in a ciguatoxic area in Lifou (Hunëtë, Loyalty Islands, New Caledonia) from March 2005 to August 2007. Sampling sites and procedures were similar to those used for the collection of *Hydrocoleum lyngbyaceum* previously described by Laurent et al., 2008. These bundled cyanobacteria were collected by hand, identified on the basis of morphological criteria and treated in the same way as *Trichodesmium*.

Table 1
Trichodesmium and benthic Oscillatoriales bloom sampling dates and locations in New Caledonia.

Reference	Dominant species[a]	Date of collection	Location	GPS
A	*T. erythraeum*	January 16, 2007	SE Lagoon, Ouinné Bay	21°59′15″S 166°41′08″E
B	*T. erythraeum*	January 18, 2007	SE Lagoon, Ouinné Bay	22°00′00″S 166°78′00″E
C	*T. thiebautii*	January 22, 2007	SE Lagoon, Ouinné Bay	22°00′00″S 166°78′00″E
D	*T. erythraeum* (50%) *T. thiebautii* (50%)	January 22, 2007	SE Lagoon, Ouinné Bay	22°00′00″S 166°78′00″E
E	*T. erythraeum*	September 24, 2007	SW Lagoon, Baie des citrons	22°17′49″S 166°26′15″E
F	*T. erythraeum*	February 18, 2008	SW Lagoon, Ricaudy Reef	22°18′44″S 166°27′34″E
G	*T. erythraeum*	February 18, 2008	SW Lagoon, Dumbéa Pass	22°21′19″S 166°17′13″E
H	*Hydrocoleum* spp.	March 15, 2005	Lifou, Loyalty Islands	20°46′00″S 167°05′33″E
I	*Oscillatoria* spp.	May 29, 2006	Lifou, Loyalty Islands	20°46′00″S 167°05′33″E
J	*Phormidium* sp., *Hydrocoleum* sp.	August 22, 2006	Lifou, Loyalty Islands	20°46′00″S 167°05′33″E

[a] Genera listed in order of abundance within the bloom sample.

2.2. Extraction

Pellets obtained from both pelagic and benthic cyanobacterial samples were extracted using solvent partition, in order to separate lipid-soluble compounds and water-soluble compounds. Freeze-dried bundles of cyanobacteria were extracted twice with methanol (1 l/100 g of freeze-dried bundle) by ultra-sonication (1 h) and agitation overnight. This extract was filtered and dried and the evaporated residue was partitioned between aqueous methanol (60%; 500 ml/100 g of freeze-dried bundle) and diethyl ether (250 ml/100 g of freeze-dried bundle). The two phases were separated and dried. The extraction data are given in Table 2. Only mouse bioassay (MBA) was performed on water-soluble extracts, whereas the lipid-soluble extracts were tested using MBA, the mouse neuroblastoma cells assay (N2A assay) and the receptor binding assay (RBA).

2.3. Mouse bioassay

The MBA is based on the careful observation of the symptoms displayed by the animals following injection of toxic extracts. All tested animals were treated under conditions which meet the

Table 2
Extraction of cyanobacteria.

Reference	Dominant species[a]	Freeze-dried bundles (g)	Fractions	
			Lipid-soluble g (%)	Water-soluble g (%)
A	*T. erythraeum*	0.275	0.014 (5.2)	0.143 (52.0)
B	*T. erythraeum*	0.288	0.008 (2.7)	0.059 (20.5)
C	*T. thiebautii*	0.543	0.049 (9.0)	0.154 (28.4)
D	*T. erythraeum* (50%) *T. thiebautii* (50%)	0.180	0.009 (5.0)	0.070 (38.9)
E	*T. erythraeum*	46.40	0.464 (1.0)	11.275 (24.3)
F	*T. erythraeum*	16.0	0.352 (2.2)	3.440 (21.5)
G	*T. erythraeum*	10.2	0.285 (2.8)	2.672 (26.2)
H	*Hydrocoleum* spp.	ND	0.142	0.335
I	*Oscillatoria* spp.	ND	0.199	0.452
J	*Phormidium* sp., *Hydrocoleum* sp.	478	1.606 (1.0)	ND

[a] Genera listed in order of abundance within the bloom sample; ND: not done.

ethical standards defined by the European Communities Council Directive of 24 November 1986 (86/609/EEC).

MBA was performed according to the method previously described by Dechraoui et al. (1999), on 20 g ± 2 g mice (OF1, Iffa-Credo, L'Arbresle, France) of either sex. The animals were allowed food and water *ad libitum*. The dried extracts were dissolved in 300 μl of phosphate buffer saline (PBS, pH 7.2) containing 0.1% Tween 80, prior to administration via intraperitoneal (i.p.) injection. The tested doses varied from 0.5 to 5.0 mg of extract/g of mouse body weight ($n \geqslant 2$; 3 concentrations depending on the extract). In total, six animals were used per extract. Control animals received 300 μl of vehicle ($n = 2$). Animal behavioral changes were observed over a period of approximately 48 h.

2.4. Mouse neuroblastoma cells assay

The N2A assay, developed by Manger et al. (1993) to detect sodium channel activators marine toxins, is based on the combined specific cytotoxicity of ouabain (O), veratridine (V) and CTXs (a site 5 sodium channel activator). The cell viability is determined using the quantitative colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, a method previously described by Mossman (1983).

Briefly, N2A cells (ATCC CCL131) were grown in RPMI-1640 medium supplemented with 1 mM sodium pyruvate, 50 μg/ml streptomycin, 50 units/ml penicillin, 25 μg/ml amphotericin B and 10% heat-inactivated fetal bovine serum. The cells were harvested with trypsin–EDTA-4Na solution (0.05% – 0.53 nM). For N2A assay, cells were seeded into tissue culture treated 96-well plates at a density of 50,000 cells/wells in 200 μl medium, 24 h prior to treatment with toxic extracts and maintained at 37 °C in a 5% CO_2 incubator.

Cells were then incubated in the presence of O (0.5 mM), V (0.05 mM) and the lipid-soluble extract ($n = 3$). Our controls consisted of (1) media + cells (standard medium; $n \geqslant 9$), of (2) media + cells + O + V (OV; $n \geqslant 9$) and of (3) media + cells + O + V + varying concentrations of brevetoxins-3 (PbTx-3) (10, 100 and 500 nM; $n = 2$). PbTx-3 is a site 5 sodium channel activator used as a model to verify the sensitivity of the cells to CTXs. The OV controls must have 80% of cell viability relative to control cells in standard medium. The viability of cells treated with extracts was estimated with respect to OV wells. For each extract, 2-fold serial dilutions in sterile water were prepared (ranging from 0.061 to 250 μg of extract per ml). These solutions (10 μl) were added in each well in a final total volume of 200 μl. After incubation at 37 °C and 5% CO_2 for 14 h, the overlaying culture media was discarded from the wells and 50 μl of MTT solution (0.8 g/l of MTT in PBS) was then added to all wells. After 1 h of incubation, 150 μl of dimethyl sulfoxide was added to dissolve the purple formazan crystals resulting from the enzymatic reduction of MTT. The microplates were further incubated for another hour. After thorough agitation, the absorbance 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 calculated as the EC_{50} values expressed in μg/ml of extract, whereby EC_{50} is defined as the effective concentration of cyanobacterial extract capable of inducing 50% of cell mortality. Sigmoidal curve fitting, EC_{50} and Hill slope values calculations were performed using GraphPad Prism version 4.01 for Windows (GraphPad Software, San Diego, California, USA).

2.5. Receptor binding assay

Lipid-soluble extracts likely to contain CTXs or CTXs-like compounds were assayed using the RBA. This test has been especially devised to detect the presence of compounds such as brevetoxins (PbTxs) and CTXs that have a differential affinity for the site 5 of the voltage-sensitive sodium channel (VSSC), purified from rat brain synaptosomes (Poli et al., 1986).

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 (Chinain et al., 1999). For all samples, two aliquots were tested in duplicates with eight dilutions of lipid-soluble extracts ranged from 2.4 to 1600 μg/ml varying according to samples. Radioactivity was determined using a Perkin Elmer Microbeta Trilux 1450 liquid scintillation counter in 2 ml Perkin Elmer Betaplate scintillation cocktail.

The RBA toxicity of lipid-soluble extracts was expressed as IC_{50} values, expressed in μg/ml of extract, whereby IC_{50} is defined as the concentration of extract capable of inducing 50% inhibition of binding of [^{3}H]PbTx-3. Sigmoidal curve fitting, Hill slopes and IC_{50} calculations were performed using GraphPad Prism version 4.01.

3. Results

3.1. Taxonomic identification of cyanobacterial samples

Samples collected from various batches of *Trichodesmium* blooms (Fig. 1a and b) reported from different locations are detailed in Table 1. Two species were identified based on morphological criteria (Janson et al., 1995). *T. erythraeum* are typically composed of trichomes colonies oriented in parallel (Fig. 1c). Cells are shorter in length (5.4–11 μm) than in width (7–11 μm) (Fig. 1d) and end cells were seen with or without a calyptra. *T. thiebautii* colonies are composed of twisted trichomes, 7–16 μm wide forming "rope-like" colonies. Commonly, the cells are twice as long as wide. The end cells often have a calyptra.

In all samples, although trichomes were often found associated with a great variety of organisms, such as copepods, bacteria, diatoms, fungi, protists, dinoflagellates and hydrozoans, cyanobacteria still represented the major component of the blooms. It is worth mentioning that no dinoflagellates known as causative agents of CFP (e.g. *Gambierdiscus*) were observed in association with these blooms of *Trichodesmium*.

Morphological analysis revealed that the cyanobacterial samples collected from benthic blooms in Lifou also belonged to the Oscillatoriales order (Table 1). The three mat-forming cyanobacteria sampled from March 2005 to August 2006 were dominated by *Hydrocoleum* spp., *Oscillatoria* spp. and a combination of *Phormidium* sp. and *Hydrocoleum* sp., respectively, three genera that are morphologically and genetically similar to *Trichodesmium* (Abed et al., 2006).

3.2. Analysis of water-soluble fractions

3.2.1. Mouse bioassay

All water-soluble extracts of *Trichodesmium* injected were toxic. Mice exhibited paralysing symptoms. Signs in mice included reduced activity and responsiveness, cyanosis of extremities,

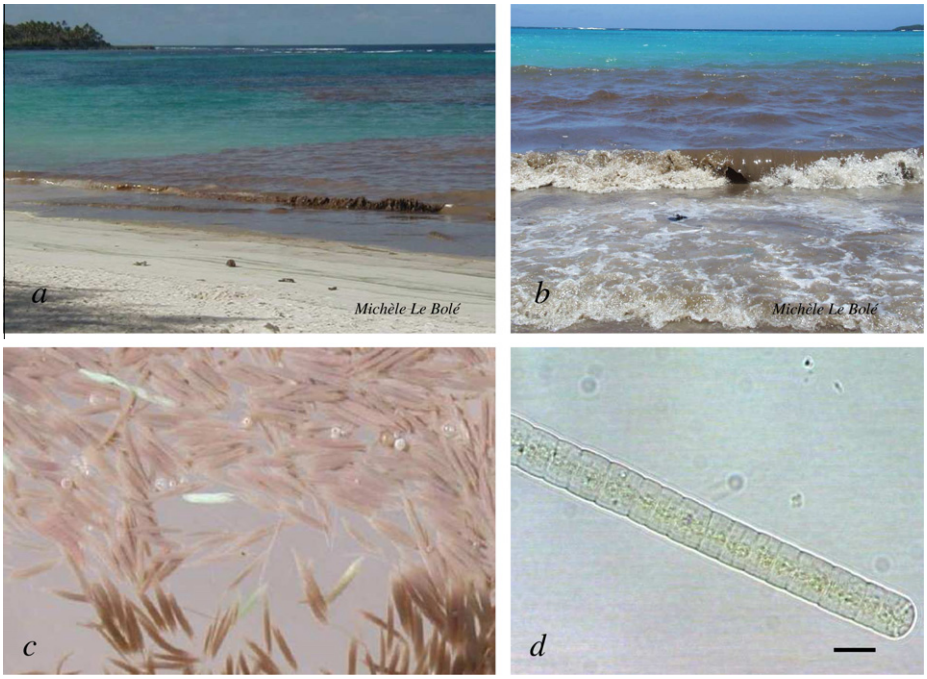

Fig. 1. *Trichodesmium erythraeum*: (a and b) Blooms of *Trichodesmium erythraeum*; (c) *Trichodesmium erythraeum* colonies and (d) microscopic view of a trichome (scale bar = 5 μm).

frequently convulsive spasms, respiratory difficulty and partial paralysis which quickly evolved to total paralysis. No salivation or lacrimation was observed. The i.p. injection of 2.5 mg of water-soluble extracts/g of mouse body weight (corresponding to roughly 12 mg of freeze-dried pellets of cyanobacteria/g of mouse body weight) killed all mice within the first 5 mn. Below this concentration, mice completely recovered despite a possible transient phase of "coma" observed during 2 h.

No differences were noted in the activity and toxic potency of *T. erythraeum* blooms collected from different locations, as well as between *T. erythraeum* and *T. thiebautii* samples.

Mice injected with benthic cyanobacteria extracts also exhibited similar symptoms (data not shown).

3.3. Analysis of lipid-soluble fractions

3.3.1. Mouse bioassay

The MBA on all lipid-soluble extracts from *Trichodesmium* samples revealed onset of quiescence, dyspnea, reduced reflex, lacrimation and hind limb paralysis over a 48 h observation period. This was also observed with the lipid-soluble extracts from the various benthic cyanobacteria samples (Laurent et al., 2008 for sample H and data not shown for samples I and J). These symptoms are similar to those observed following exposure to pure CTXs, whereby the characteristic symptoms are signs of inactivity, severe diarrhoea, dyspnea, cyanosis, piloerection, tremors, paralysis and staggering gait (Lehane and Lewis, 2000).

3.3.2. N2A assay

To detect the presence of compounds acting like CTXs, lipid-soluble extracts of cyanobacteria were assayed in N2A assay. All lipid-soluble extracts tested were found to enhance the ouabain/veratridine-induced activation of the VSSC (Table 3). EC_{50} values ranged from 149 to 218 μg of extract per ml (μg/ml) with mean values around 165 μg/ml. Typical sigmoidal dose–response curves of *Trichodesmium* lipid-soluble extracts exhibited absolute values of Hill slope ranging from 1.7 to 8.6 (r^2 = 0.950 to 0.990). In contrast, experiments with sample E (Table 3) yielded a very steep dose–response curve with the greatest absolute value of 22.3 (r^2 = 0.979), which must probably reflects a matrix effect rather than a true biological effect.

No significant or major differences were observed between the EC_{50} values of *Trichodesmium* samples from various geographical locations (samples B, E, F and G, Table 3), or from different sampling seasons (samples E and F, G, Table 3).

In the same way, the toxicity of *T. thiebautii* and *T. erythraeum* did not differ significantly (sample C versus samples E, F or G, Table 3). In contrast, the toxicity of the "mixed" *T. thiebautii*/*T. erythraeum* bloom was significantly lower than the ones of the respective monospecific blooms (sample D versus samples B, C, E, F or G, Table 3).

These EC_{50} values have been tentatively compared to those obtained from benthic blooms of *Oscillatoria* spp (sample I, Table 3) and *Phormidium* spp. mixed with *Hydrocoleum* spp. (sample J, Table 3) collected in Lifou. These two samples showed a higher toxicity than their pelagic counterparts of around 3- to 6-fold (55 and 35 μg/ml respectively).

3.3.3. Receptor binding assay

To further assess whether the cytotoxic effect observed in the N2A assay was mediated through the activation of site 5 of the VSSC, lipid-soluble extracts of *Trichodesmium* samples were also assayed in RBA experiments.

Sigmoidal dose–response curves of *Trichodesmium* lipid-soluble extracts are presented in Fig. 2. Samples A to C showed curves with the lowest absolute Hill slopes values, ranging from 0.51 to 0.67 for (r^2 = 0.965 to 0.986), while those of samples D to J ranged from 1.02 to 1.70 (r^2 = 0.970 to 0.992). Our results demonstrated that all *Trichodesmium* lipid-soluble extracts contain compounds capable of binding to site 5 of the VSSC (Fig. 2), but in varying concentrations (Table 3).

RBA results summarized in Table 3 showed that among *Trichodesmium* blooms collected in January 2007 (A to D samples, Table 3), extract from *T. thiebautii* (sample C, Table 3) was less

Table 3
Results of mouse neuroblastoma cells assay (N2A assay) and receptor binding assay (RBA) obtained from lipid-soluble extracts of *Trichodesmium* samples and other benthic Oscillatoriales from Lifou (Loyalty Island, New Caledonia).

Reference	Location	Date	Dominant species[a]	N2A assay EC_{50} ± SEM (μg of extract/ml)	RBA IC_{50} ± SEM (μg of extract/ml)
A	*SE lagoon, Ouinné*	January 16, 2007	*T. erythraeum*	ND	570.6 ± 105.6
B	*SE lagoon, Ouinné*	January 18, 2007	*T. erythraeum*	149.8 ± 7.5	287.5 ± 13.4
C	*SE lagoon, Ouinné*	January 22, 2007	*T. thiebautii*	168.7 ± 8.4	997.9 ± 121.3
D	*SE lagoon, Ouinné*	January 22, 2007	*T. erythraeum*, *T. thiebautii*	218.8 ± 10.9	309.6 ± 71.3
E	*Nouméa, Baie des Citrons*	September 24, 2007	*T. erythraeum*	165.2 ± 8.3	54.9 ± 0.4
F	*SW lagoon, Ricaudy reef*	February 18, 2008	*T. erythraeum*	167.5 ± 8.4	41.08 ± 9.3
G	*SW lagoon, Dumbéa pass*	February 18, 2008	*T. erythraeum*	165.8 ± 8.3	ND
H	*Lifou, Loyalty Island*	March, 2005	*Hydrocoleum* spp.	ND	244.4 ± 11.8
I	*Lifou, Loyalty Island*	May, 2006	*Oscillatoria* spp.	55 ± 3	ND
J	*Lifou, Loyalty Island*	August, 2006	*Phormidium* sp., *Hydrocoleum* sp.	35 ± 4	82.6 ± 7.2

[a] Genera listed in order of abundance within the bloom sample; ND: not done.

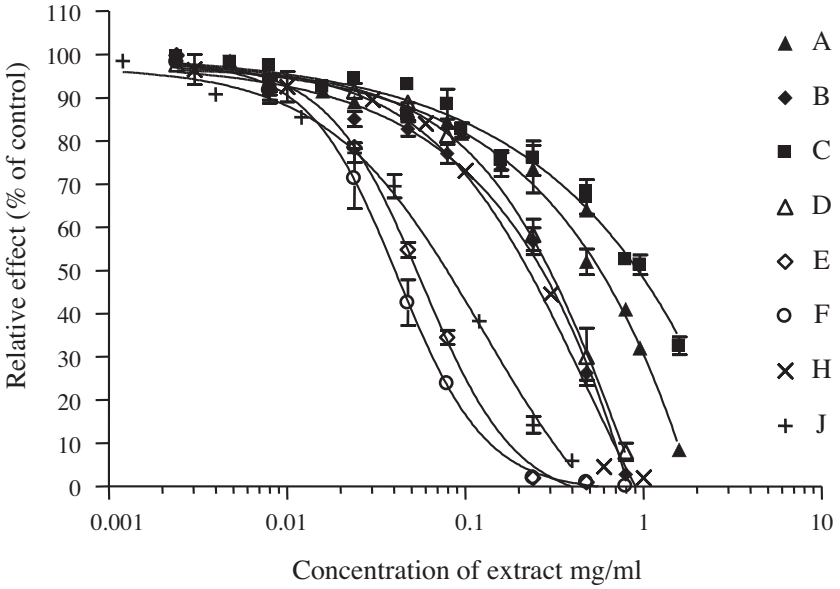

Fig. 2. Inhibition curves of lipid-soluble extracts from cyanobacteria samples collected in New Caledonia on rat brain membrane preparation using the RBA. [^{3}H]PbTx-3 specific binding was measured following the protocol developed in the Section 2. Each point represents the mean (±SEM) of two to four experiments. Prism calculated IC_{50} values calculated from sigmoidal dose–response (variable slope) regression which are reported in Table 3. The letters correspond to cyanobacteria samples reported in Table 3: A = *T. erythraeum* (January 16, 2007), Ouinné; B = *T. erythraeum* (January 18, 2007), Ouinné; C = *T. thiebautii* (January 22, 2007), Ouinné; D = *T. thiebautii* & *T. erythraeum* (January 16, 2007), Ouinné; E = *T. erythraeum* (September 24, 2007), Nouméa, Baie des Citrons; F = *T. erythraeum* (February 18, 2007), SW lagoon, Ricaudy Reef; H = *Hydrocoleum* spp. (March, 2005), Loyalty Island, Lifou; J = *Phormidium* sp. & *Hydrocoleum* sp. (August, 2005), Loyalty Island, Lifou.

potent in binding to sodium channel than those from *T. erythraeum* (samples E and F, Table 3). Regarding the *T. erythraeum* blooms, RBA value of sample F collected in February 2008 was almost fifteen-fold higher than sample A collected in January 2007.

Finally, it is interesting to note that according to RBA results, the mixed *Phormidium* and *Hydrocoleum* bloom collected in Lifou in August 2006 (sample J, Table 3) appeared less potent than the two *T. erythraeum* blooms collected in Baie des Citrons (sample E, Table 3) and Ricaudy reef (sample F, Table 3), respectively, which is clearly in contrast with the corresponding N2A assay results (Table 3).

4. Discussion

As observed during our surveys, the cyanobacteria of the genus *Trichodesmium*, most notably the species *T. erythraeum*, are the most prevalent pelagic cyanobacteria found in the New Caledonia lagoon during the months of September to March. This observation is in agreement with previous report by Rodier and Le Borgne (2008, 2010).

In order to determine if blooms of these cyanobacteria could have an ecotoxicological impact and consequently be potential harm to human consumers, we decided to test various cyanobacterial blooms samples for their toxicity using different bioassays (MBA, N2A assay and RBA).

4.1. Toxicity analysis

Results presented in this study support the co-occurrence of paralysing toxins and CTXs-like compounds in all samples of *Trichodesmium* collected in the New Caledonia lagoon from 2007 to 2008. This has also been observed with the benthic species analyzed in the present study.

The MBA assays demonstrated paralysing response of the water-soluble extracts from our collected *Trichodesmium* spp. The

observed symptoms (respiratory difficulty, partial paralysis, total paralysis, severe convulsions, death by respiratory failure) suggest the presence of PSTs or anatoxin-a, homoanatoxin-a or anatoxin-a (s) in these extracts, although evidence for the presence of such toxins remains to be unmistakably substantiated by GC–MS and/or LC–MS analysis. These analyses are currently underway on the water-soluble extracts obtained during this study.

Symptoms observed in MBA assays following the administration of lipid-soluble extracts of *Trichodesmium*, in particular hind limb paralysis and dyspnea are in favor of the presence of CTXs-like compounds in these extracts. However, cyanosis, another classical symptom of CTXs in MBA could not be observed in our assays.

The presence of such compounds in *Trichodesmium* extracts were further confirmed using N2A assay and RBA, which are routinely used to quantify CTXs activity (Manger et al., 1993, 1995; Bottein-Dechraoui et al., 2008). As a matter of fact, the RBA toxicity values of *T. erythraeum* samples collected in September 2007 and February 2008 clearly denote a stronger displacement of the [^{3}H]PbTx-3 than those collected in January 2007.

The same observation also applies to *Hydrocoleum* samples from Lifou, suggesting that these CTXs-like compounds are not specific to pelagic cyanobacteria but are also present in benthic cyanobacteria that are morphologically and genetically similar to *Trichodesmium* (Abed et al., 2006; Laurent et al., 2008).

In addition, RBA results regarding the toxicity of *T. erythraeum vs T. thiebautii* and of *T. erythraeum* blooms toxicity in 2007 vs. 2008 clearly indicates the existence of both inter- and intra-specific variability in toxin production within this order (Qiqin et al., 1997; Thacker and Paul, 2004). Besides this variability due to genetic factors, the toxinogenic potential of *Trichodesmium* species is also known to depend on the stimulation by external abiotic (temperature, light, wind...) or biotic factors (grazing, associations with bacteria) (Guo and Tester, 1994; Qiqin et al., 1997; Thacker and Paul, 2004; Negri et al., 2004; Wiegand and Pflugmacher, 2005).

It is also interesting to note that discrepancies were observed between N2A assay and RBA results. In particular, using RBA, it was possible to observe striking variations in the toxicity of the different *T. erythraeum* blooms successively tested in this study. In contrast, no major differences were observed between these same samples using the N2A assay. Such discrepancies may be explained by the differences inherent to the principles of these two tests.

The low toxicity for some extracts recorded in N2A assay in absence of sodium channel potentializers OV (data not shown) may suggest a matrix effect or the co-occurrence of sodium channel toxins or even other types of toxins with different target specificity leading to cellular cytotoxicities (Carmichael et al., 1997; Van Dolah, 2000; Van Apeldoorn et al., 2007). Therefore, utilization of the two *in vitro* tests, N2A assay and RBA, in this study provides complementary information that aids in the identification of toxin of cyanobacteria.

Thus, it can be hypothesized that the differences in the neurotoxicity of *Trichodesmium* observed between the previous studies may depend on (i) the species and/or on the strains, (ii) the form of experimentation conducted on intracellular toxins and intact cells, and (iii) are inherent to the specificity and the sensibility of the test used (Hawser et al., 1992; Hahn and Capra, 1992; Endean et al., 1993; Guo and Tester, 1994; Sellner, 1997).

The co-occurrence of CTXs-like and paralysing toxins in *Trichodesmium* extracts observed in the present study are coherent with previous observations by Endean et al. (1993).

Finally, all our assays were conducted on crude and thus rather "impure" samples, therefore, the possible contribution of toxicity of other microorganisms in our MBA, N2A assay and RBA cannot be completely ruled out. The successful culturing of *Trichodesmium* spp. strains isolated from the field in laboratory axenic conditions, will hopefully allow proper ecotoxicological studies avoiding interferences from other microorganisms (Chen et al., 1996).

4.2. Impact of grazing on the food-web

Trichodesmium colonies constitute a living habitat for various types of marine organisms (Sellner, 1997). They are also used as a nutritional substrate by certains species showing tolerance to the concentrated toxins, like the pelagic copepods, *Macrosetella gracilis* and *Miracia efferata* (O'Neil and Roman, 1992; Sellner, 1997). Therefore, the toxins likely to be produced by these cyanobacteria can potentially enter the food chain.

As a matter of fact, cases of ciguatera intoxications due to the ingestion of mullets (*Mugilidae*), known to graze on *Trichodesmium* trichomes have been reported in a local epidemiological survey (personal communication from local fishermen).

Similarly, Endean et al. (1993) also demonstrated that the toxins produced by *T. erythraeum* were indistinguishable from those present in the flesh of the narrow-barred Spanish mackerel *Scomberomorus commersonii*, frequently implicated in CFP outbreaks.

Although the link between primary producers to subsequent trophic levels in this ecotoxicological phenomenom food chain still needs to be clarified, the occurrence of CTXs-like compounds in *Trichodesmium* support the idea that the grazing of highly toxic blooms of this cyanobacteria may lead to possible CFP risk in the New Caledonia lagoon. Further surveys are needed to clearly establish this link.

4.3. Ecological impact of Trichodesmium blooms

There are no extensive reports of fish or shellfish mortality or others forms of intoxications specifically associated with blooms of *Trichodesmium* spp. These blooms are seen as natural components of marine ecosystems. Yet, such blooms have been occasionally suspected in adverse effects or mortality episodes of the marine life, though it is still unclear whether these instances were simply due to poor water quality or to actual toxic activity (Sellner, 1997; Negri et al., 2004). Indeed, *Trichodesmium* blooms have been shown to modify both the concentration of nutrients and the penetration of light (Krishnan et al., 2007). Negri et al. (2004) have linked a massive mortality of pearl oysters within aquaculture farms of Australia to the potential anoxic effects of a *T. erythraeum* bloom. However the authors could not observe any neurotoxic activity either in mice or in *in vitro* saxiphilin and sodium channel assays.

5. Conclusion

Taken all together, these results are clearly in favor of: (i) the co-occurrence of lipid-soluble toxins (i.e. CTXs-like toxins) and water-soluble paralysing toxins in *T. erythraeum* and *T. thiebautii*, two species of *Trichodesmium* spp. that are widespread in New Caledonia waters, and (ii) the possible contribution of these pelagic cyanobacteria to the ciguatera food chain.

A more comprehensive study regarding the isolation and characterization of the toxins involved and their subsequent impact on the food-web is currently underway in our laboratory.

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