NRC·CNRC

Increasing Prevalence of Marine and Freshwater toxins: A Global Measurement Challenge

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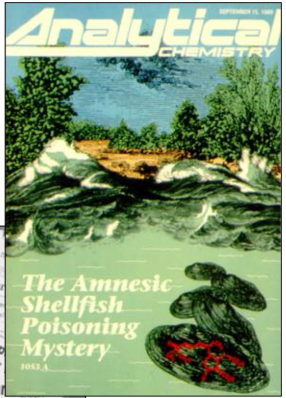
National Research Council Canada, Metrology Research Center
October 19, 2024

Marine algal toxins – commonly associated with “red tide”

- Produced by marine dinoflagellates (i.e. phytoplankton) and diatoms and accumulate in filter feeders.
- Established worldwide regulations and shellfish safety testing.



Impact of Shellfish Poisoning

- Hundreds of deaths and thousands of illnesses occur each year globally.
- The estimated global economic impact of shellfish toxins is estimated at 4 billion US dollars per year.*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Crab pots lie empty, boats idled as toxic algae stalls a San Francisco tradition

In Case You Missed It

Imported Dungeness crabs are for sale at Fisherman's Wharf as the local crab season is closed. (Eric Risberg / Associated Press)

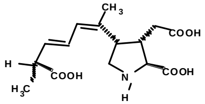
Summer 2015 – Domoic acid impacts multiple fisheries and marine mammals across west coast of North America

* http://www.oceansatlas.org/unatlas/uses/uneptextsph/wastesph/2571gs71041health.html

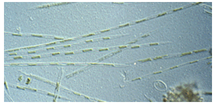
Paralytic Shellfish Poisoning Incidents – Saxitoxins: A Global Problem

Marine Algal Toxins – Diverse Challenge

Amnesic Shellfish Poisoning

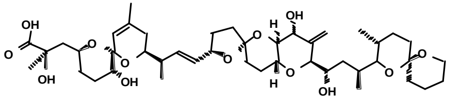


DomoicAcid



Pseudonitzschia spp.

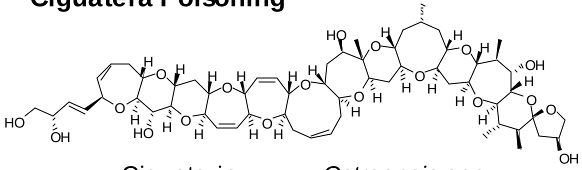
Diarrhetic Shellfish Poisoning



OkadaicAcid

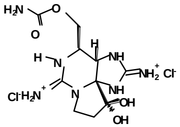
Dinophysis spp.

Ciguatera Poisoning



Ciguatoxin *Ostreopsis spp.*

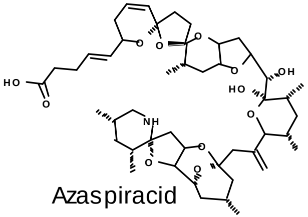
Paralytic Shellfish Poisoning



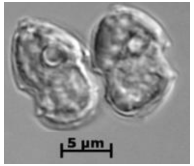
Saxitoxin

Alexandrium tamarense

Azaspiracid Shellfish Poisoning

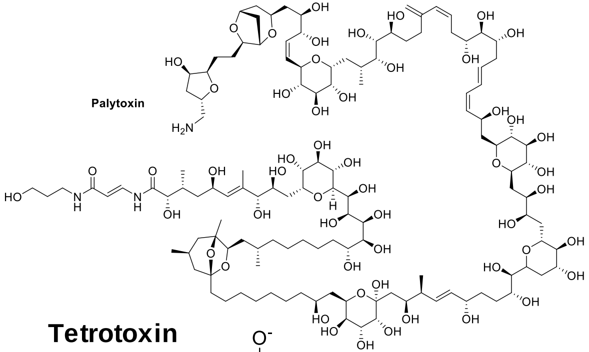


Azaspiracid



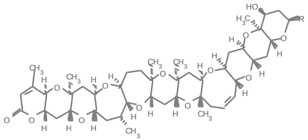
Azadinium spinosum

Palytoxin & Ovatoxins



Palytoxin

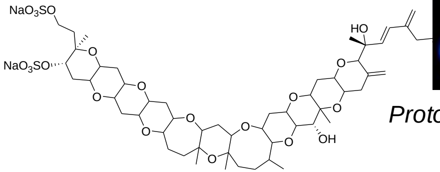
Neurotoxic Shellfish Poisoning

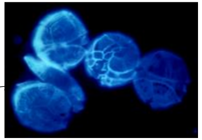


Brevetoxin

Karenia brevis

Yessotoxins

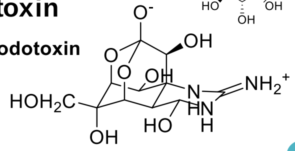




Protoceratium reticulatum

Tetrotoxin

Tetrodotoxin



Cyanobacterial Toxins

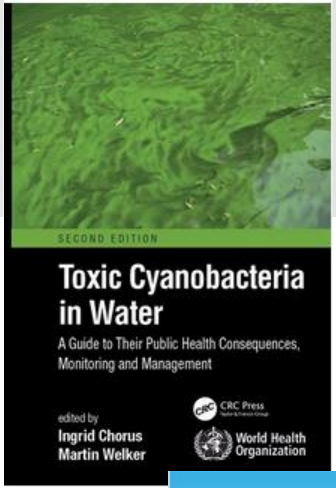
- Originate primarily in freshwater systems
- Presence in drinking/recreational water and fish considered an emerging health concern
- Persistent global problem increasing in temperate regions due to climate change, e.g. increasing occurrence in northern Canadian communities previously unaffected
- Measurement essential for regulatory monitoring, public health and industry protection, international trade, etc.

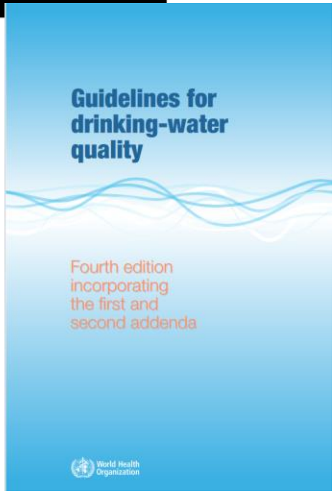
WHO Guidelines

Updated in 2022 to include short- and long-term exposure guidelines in drinking and recreational waters based on available toxicology and occurrence data.

<table>
<tr><th>Class</th><th>Short-term (µg/L)</th><th>Long-term (µg/L)</th></tr>
<tr><td>microcystins</td><td>12</td><td>1</td></tr>
<tr><td>anatoxins</td><td colspan="2">30</td></tr>
<tr><td>saxitoxins</td><td colspan="2">3</td></tr>
<tr><td>cylindrospermopsins</td><td>3</td><td>0.7</td></tr>
</table>

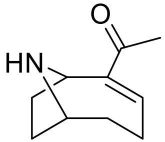
Still broad range of regulations and guidelines across different jurisdictions globally.



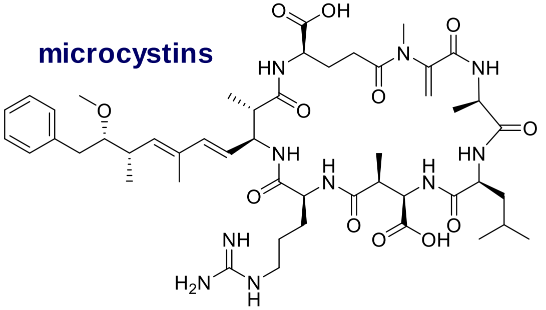


Cyanotoxin Measurement Challenges

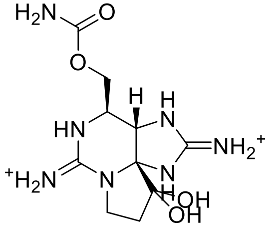
- Diversity of cyanotoxin classes
  - Varying polarity and toxic mechanism
  - Within-class chemical diversity (e.g. over 300 microcystins)
- Analytical techniques
  - Chemical, immunochemical, enzymatic and genetic assays all measure different properties
- Limited resources
  - Reference materials
  - Methods for simultaneous analysis of multiple classes



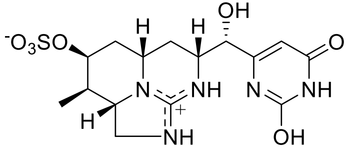
anatoxins



microcystins



saxitoxins



cylindrospermopsins

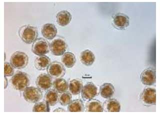
Toxin CRM Process: Overview

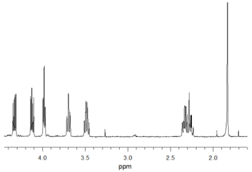
Outbreak

- Illness
- Public Health link to shellfish/fish consumption

Toxin Discovery

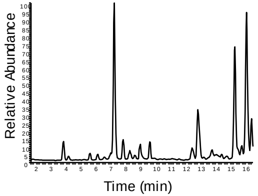
- Bioassay guided fractionation
- Chemical characterization
- Causative agent
- Transformations





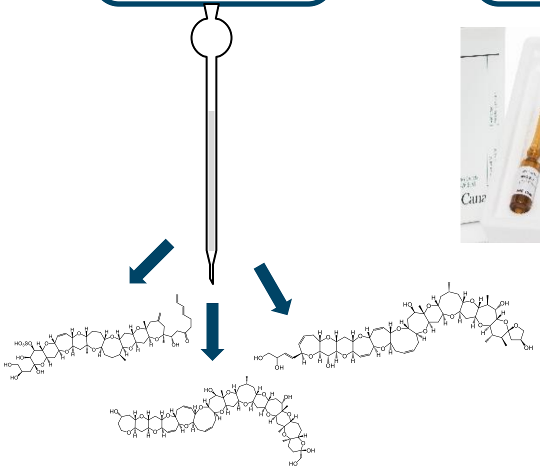
Analytical Tools

- Qualitative measurement
- HRMS screening
- Triple-quad quantitative measurements



Isolation

- Fractionation procedures
- Purification
- Feasibility studies



CRM Production

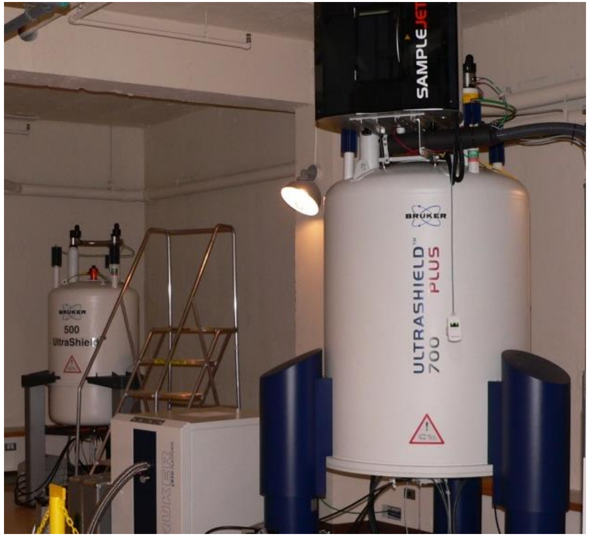
- Ampouling
- Homogeneity
- Stability
- Value assignment
- All under ISO 17034

Challenges in the development of calibration solutions

- Marine biotoxins and cyanotoxins are rare and difficult to acquire
- Material obtained through laboratory cultures or contaminated samples
- Sometimes only 2-5 mg for entire CRM production
- In the absence of a primary standard, need to demonstrate traceability through potentially unrelated compound

Biotoxin Primary Methods

1H- Quantitative Nuclear Magnetic Resonance Spectroscopy (qNMR)



- Equal response from protons regardless of structure
- Widely applicable to any H-containing molecule
- Non-destructive, can analyze actual CRM stock solution at mM-level prior to dilution
- Traceability from certified reference materials using external calibration

Anal. Chem. **2005,** *77,* 3123−3131

Quantitative ¹H NMR with External Standards: Use in Preparation of Calibration Solutions for Algal Toxins and Other Natural Products

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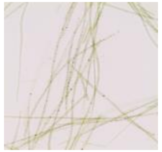
NRC Biotoxin Metrology – dedicated team to marine and freshwater toxin measurements

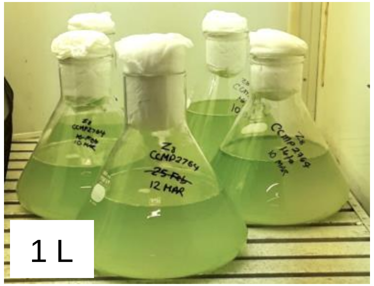
- Analytical laboratories in Halifax and unique Marine Research Station in Ketch Harbour, Nova Scotia that facilitates large scale cultures
- 16 scientists, 1 researcher emeritus, supporting operations staff, visiting workers and students, led by Dr. Pearse McCarron
- Range of research and measurement science activities in support of algal biotoxin analysis
- Supports nearly 50 calibration solution and matrix biotoxin CRMs, which are distributed globally (www.nrc.ca/crm)

Halifax, Nova Scotia

Ketch Harbour, Nova Scotia

Phytoplankton and cyanobacteria culturing to acquire biomass





Some cultures are not amenable to scale-up and are limited to laboratory-scale

NRC Brite-Box systems allow for large-scale cultures with automated lighting and feeding

Calibration Solution CRMs

- Highly pure toxin (low mg amounts)
- Accurate dilutions in ampoules (low µg/mL)
- Primary method value assignment (e.g. qNMR)
- Prepared in accordance with ISO 17034, 17035
- Establish traceability (key for matrix CRMs)
- NRC CRMs for range of cyanotoxins
  - microcystins (5 analogues)
  - nodularin-R
  - anatoxin-a
  - cylindrospermopsin
  - saxitoxins (15 analogues)

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Certificate of Analysis

Certified Reference Material

CRM-MCLA (Lot# 20210128)

Certified Calibration Solution for Microcystin-LA

Microcystin-LA (MC-LA) is a cyclic peptide toxin produced by freshwater cyanobacteria [1] that has been associated with domestic and wild animal poisonings and poses a threat to human health through contamination of drinking water supplies [2]. CRM-MCLA is a certified calibration solution of MC-LA in aqueous methanol (1:1, v/v), designed to aid in the identification and quantitation of MC-LA.

Table 1: Certified concentration and uncertainty for CRM-MCLA.

Compound	µg/g	µg/mL (15 - 30 °C)	µmol/L (15 - 30 °C)
Microcystin-LA	5.07 ± 0.20	4.69 ± 0.19	5.15 ± 0.21

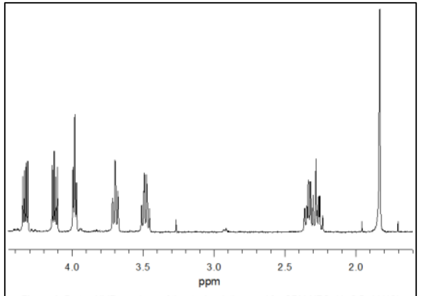
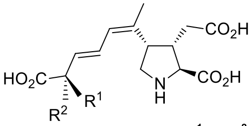
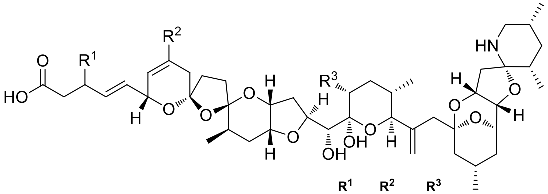


Figure 1: Proton NMR spectrum of the stock solution used for CRM-NEO-d in 0.5mM HCl

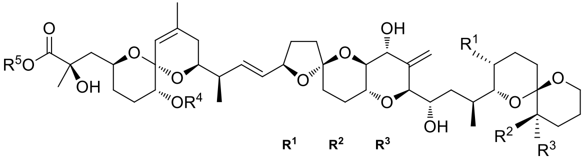
Multi-analyte material: CRM-FDMT1



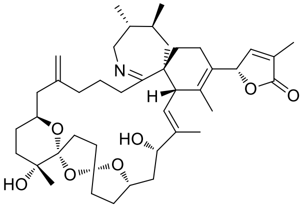
	R^1	R^2
Domoic acid	CH_3	H
5'-epi-Domoic acid	H	CH_3



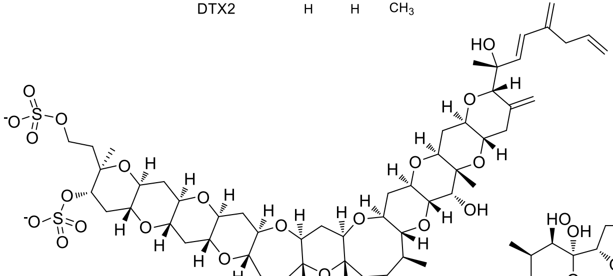
	R^1	R^2	R^3
Azaspiracid-1	H	H	CH_3
Azaspiracid-2	H	CH_3	CH_3
Azaspiracid-3	H	H	H



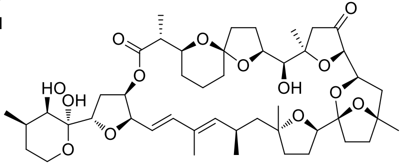
	R^1	R^2	R^3
Okadaic acid	CH_3	H	H
DTX-1	CH_3	CH_3	H
DTX2	H	H	CH_3



13-Desmethyl-spirolide C



Yessotoxin



Pectenotoxin-2

Certificate of Analysis

NRC·CNRC Certified Reference Material

CRM-FDMT1 (Lot # 20070717)

Freeze-dried Mussel Tissue Certified Reference Material for Multiple Marine Toxins

Marine algal toxins can accumulate in filter-feeding shellfish to levels that are harmful to human health. Monitoring for the presence of these toxins is required to protect consumers and the seafood industry [1,2]. CRM-FDMT1 is a freeze-dried mussel tissue (*Mytilus edulis*) containing toxins from six major groups of shellfish toxins. CRM-FDMT1 was prepared by blending contaminated mussel tissues and fortifying with cultured algae and purified toxins [3]. Certified values and expanded uncertainties (U_{CRM}) have been established for domoic acid, azaspiracid-1, -2 and -3, okadaic acid, dinophysistoxin-1 and -2, yessotoxin, pectenotoxin-2, and 13-desmethylspirolide C (Tables 1 and 2). Information values have also been assigned for a number of additional analytes from each toxin group (Tables 4 and 5).

Table 1: Certified concentration values and associated uncertainties for CRM-FDMT1.

Compound	Concentration[1] (mg/kg)
Domoic acid (DA + 5'-*epi*-DA)	126 ± 10
Azaspiracid-1 (AZA1 + 37-*epi*-AZA1)	4.10 ± 0.40
Azaspiracid-2 (AZA2 + 37-*epi*-AZA2)	1.13 ± 0.10
Azaspiracid-3 (AZA3 + 37-*epi*-AZA3)	0.96 ± 0.10
Okadaic acid (OA)	1.59 ± 0.18
Dinophysistoxin-1 (DTX1)	0.68 ± 0.07
Dinophysistoxin-2 (DTX2)	3.57 ± 0.33
Yessotoxin (YTX)	2.49 ± 0.28
Pectenotoxin-2 (PTX2)	0.66 ± 0.06
13-desmethylspirolide C (13-desMe-SPX C)	2.70 ± 0.26

[1] Certified values are based on mass of the freeze-dried powder as received.

Period of validity: 3 years from date of sale
Storage conditions: -12 °C or below

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Canada

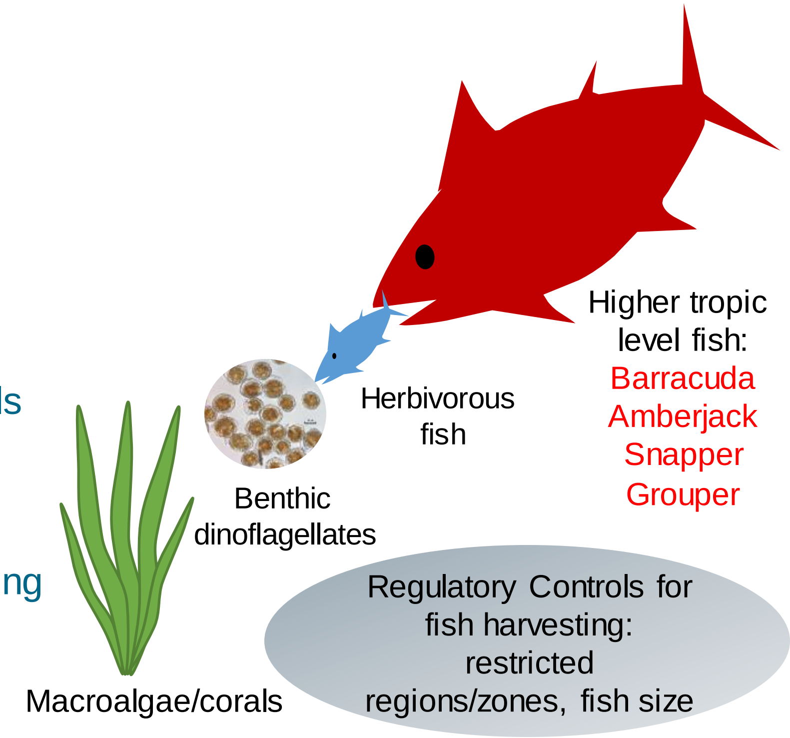
Case study: Ciguatoxin – was exclusively a Caribbean issue and now appearing in northern waters with climate change

- Linked to consumption of large fin-fish: amberjack, grouper, snapper, barracuda in tropical & subtropical regions
  - > 425 fish species linked to ciguatera poisoning

- 50,000 – 500,000 (est.) people affected annually

- Potent sodium channel activator – depolarization of nerve cells
  - Symptoms: gastrointestinal, neurological, cardiovascular
    - Generally resolve in weeks, but can last months/years

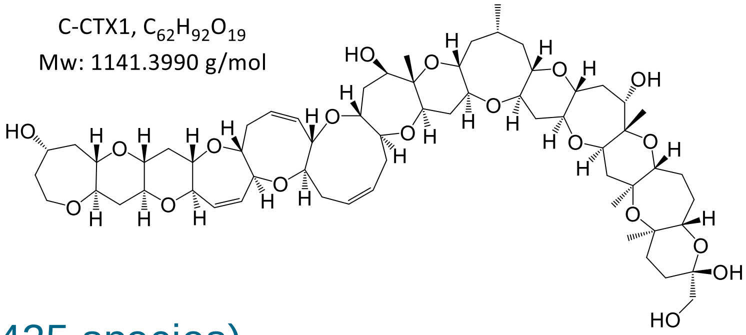
- Increasing concern worldwide: imported fish, travellers, shipping crews, climate change, increasing water temperatures, etc.



Analytical Challenges of CTXs

C-CTX1, $C_{62}H_{92}O_{19}$

Mw: 1141.3990 g/mol



- Lack of reference materials for positive confirmation
- Variety of CTX analogues (regional distribution)
- Variety of fish species, trophic levels, other seafood (>425 species)
- Limited validated analytical approaches with diagnostic features
- Poor ionization efficiency, variation in adduct formation, on-column epimerization/poor peak shape (C-CTXs)

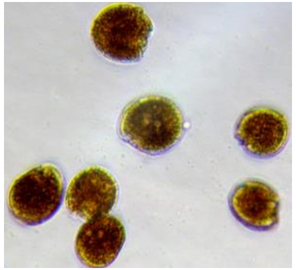
International collaboration (Canada, USA, Norway) to identify source of ciguatoxin

Collection of *Gambierdiscus* spp. from regions surrounding USVI, Caribbean, etc.

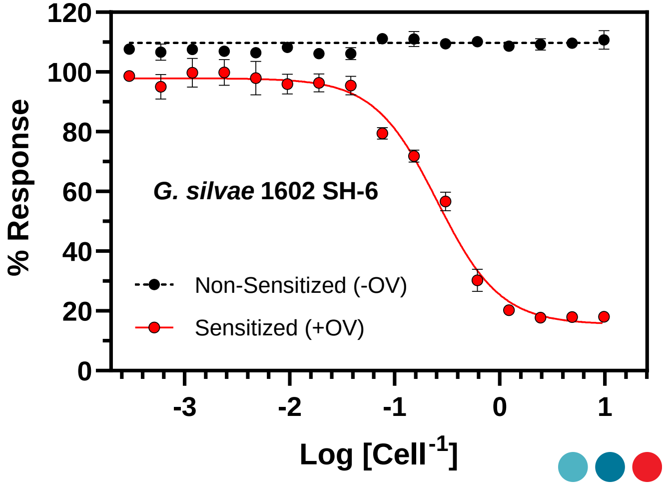
Monocultures established and maintained

Screened by N2a for CTX-like activity

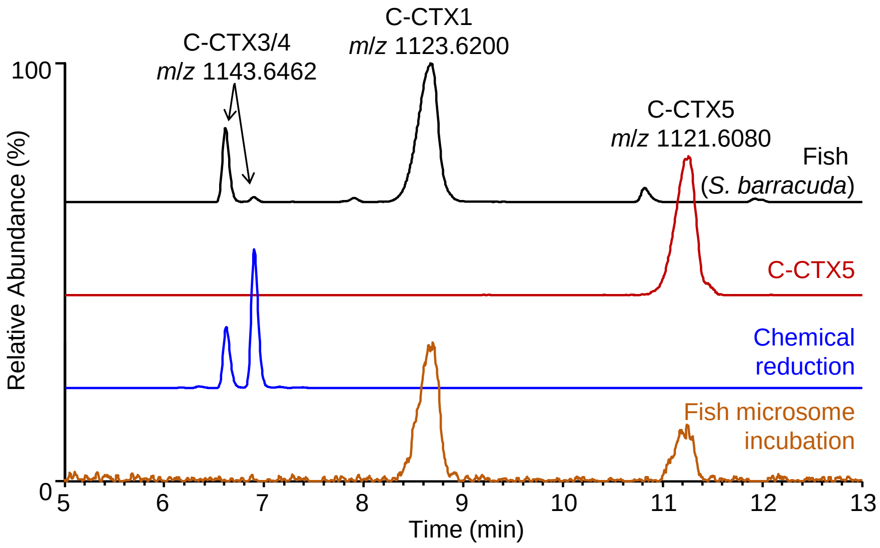
Identification of highly toxic strain off coast of St. Thomas

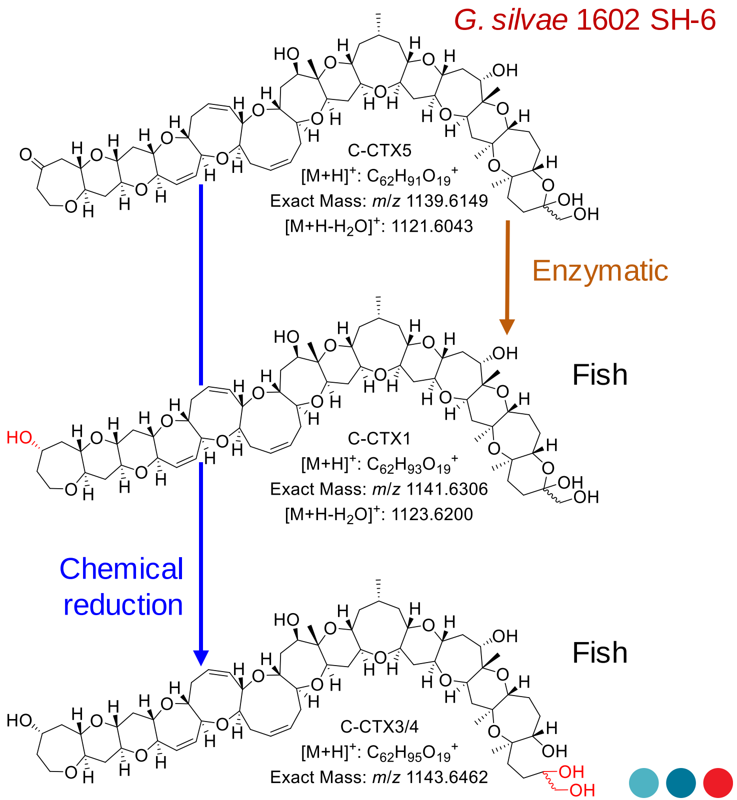


Gambierdiscus silvae 1602 SH-6; 1.5 pg CTX3C eq./cell



Confirmation of C-CTX5 structure





Identification of the source of Caribbean ciguatoxin after nearly 30 years of research in this area

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journal homepage: www.elsevier.com/locate/chemosphere

Check for updates

Algal ciguatoxin identified as source of ciguatera poisoning in the Caribbean

Elizabeth M. Mudge[a,*], Christopher O. Miles[a], Lada Ivanova[b], Silvio Uhlig[b], Keiana S. James[c,d], Deana L. Erdner[e], Christiane K. Fæste[b], Pearse McCarron[a], Alison Robertson[c,d,**]

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Developing Ciguatoxin Reference Materials

G. silvae: Growth Conditions

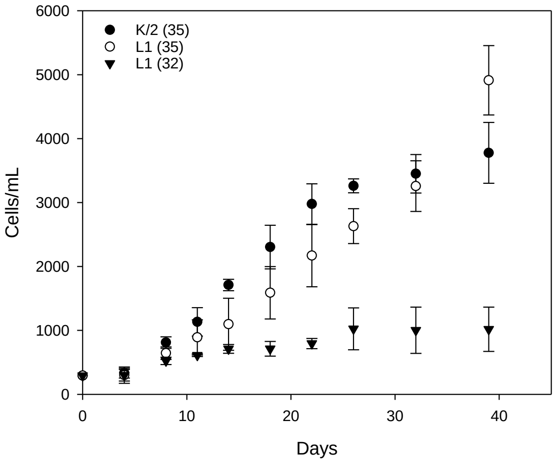
Growth trials in different media:

L1 – 32 ‰ (commonly used in-house media)

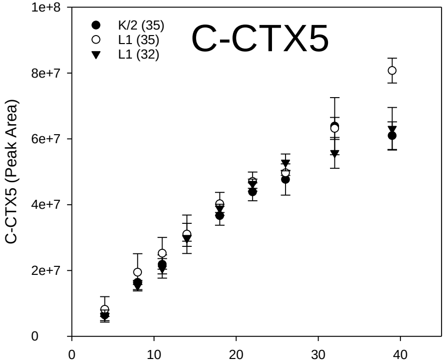
L1 – 35 ‰ (adjusted with Instant Ocean)

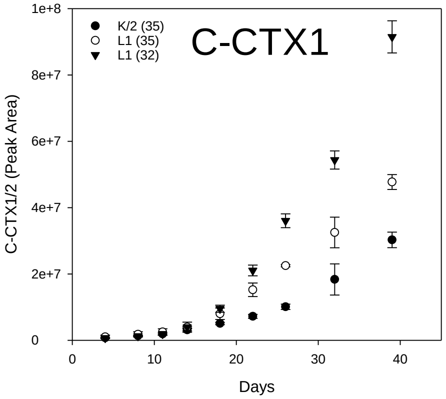
K/2 – 35 ‰ (collaborator recommendation)

Growth Curves



Toxin Production





Thank you

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