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

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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.*

* 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
Domoic Acid
Pseudonitzschia spp.

Paralytic Shellfish Poisoning
Saxitoxin
Alexandrium tamarense

Neurotoxic Shellfish Poisoning
Brevetoxin
Karenia brevis

Diarhetic Shellfish Poisoning
Okadaic Acid
Dinophysis spp.

Azaspiracid Shellfish Poisoning
Azaspiracid

Ciguatera Poisoning
Ciguatoxin
Ostreopsis spp.

Palytoxin & Ovatoxins

Yessotoxins

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>
<thead>
<tr>
<th>Class</th>
<th>Short-term (µg/L)</th>
<th>Long-term (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>microcystins</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td>anatoxins</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>saxitoxins</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>cylindrospermopsins</td>
<td>3</td>
<td>0.7</td>
</tr>
</tbody>
</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
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

Time (min)

Relative Abundance
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

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

*Ian W. Burton, Michael A. Quilliam, and John A. Walter*

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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)
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)
Multi-analyte material: CRM-FDMT1

Certificate of Analysis

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. 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 [5]. Certified values and expanded uncertainties (U(95)) have been established for domoic acid, azaspiracid-1, -2, and -3, okadaic acid, diophystoxin-1 and -2, yessotoxin, pectenotoxin-2, and 13-desmethyspirioide 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

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (µg/g)</th>
</tr>
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<tbody>
<tr>
<td>Domoic acid (DA + 5-epiDA)</td>
<td>126 ± 10</td>
</tr>
<tr>
<td>Azaspiracid-1 (AZA1 + 3'-epiAZA1)</td>
<td>4.15 ± 1.40</td>
</tr>
<tr>
<td>Azaspiracid-2 (AZA2 + 3'-epiAZA2)</td>
<td>1.13 ± 0.10</td>
</tr>
<tr>
<td>Azaspiracid-3 (AZA3 + 3'-epiAZA3)</td>
<td>0.96 ± 0.10</td>
</tr>
<tr>
<td>Okadaic acid (OA)</td>
<td>1.59 ± 0.16</td>
</tr>
<tr>
<td>Diophystoxin-1 (DTX1)</td>
<td>0.69 ± 0.07</td>
</tr>
<tr>
<td>Diophystoxin-2 (DTX2)</td>
<td>3.57 ± 1.33</td>
</tr>
<tr>
<td>Yessotoxin (YTX)</td>
<td>2.46 ± 0.28</td>
</tr>
<tr>
<td>Pectenotoxin-2 (PTX2)</td>
<td>0.65 ± 0.06</td>
</tr>
<tr>
<td>13-desmethyspirioide C (13-desMe-SPI C)</td>
<td>2.70 ± 0.26</td>
</tr>
</tbody>
</table>

*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

NRC-CNRC
Canada

15
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

- 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
Confirmation of C-CTX5 structure

- **G. silvae 1602 SH-6**

- **Enzymatic**
  - Chemical reduction
  - Fish microsome incubation

- **Chemical reduction**
  - Fish microsome incubation

- **Fish**
  - (S. barracuda)

- **Relative Abundance (%)**
  - C-CTX5
    - m/z 1121.6080
  - C-CTX3/4
    - m/z 1143.6462
  - C-CTX1
    - m/z 1123.6200

- **Time (min)**
  - 0 5 6 7 8 9 10 11 12 13

- **Confirmation of C-CTX5 structure**
Identification of the source of Caribbean ciguatoxin after nearly 30 years of research in this area

Algal ciguatoxin identified as source of ciguatera poisoning in the Caribbean

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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**

**C-CTX5**

**C-CTX1**
Thank you

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