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# 20 Years of HAB Research with the Help of Mass Spectrometry

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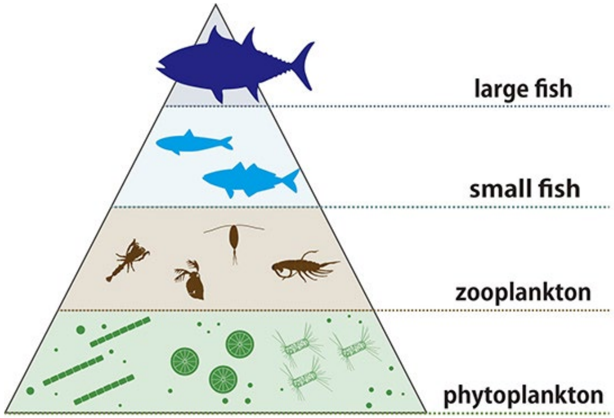
Bernd Krock, IADO, Bahía Blanca, 15th October 2024

# Introduction



- Marine toxins = Phycotoxins are toxic chemicals produced by photosynthetic plankton-species
- Dinoflagellates are the principle producers of phycotoxins
  →Also toxigenic diatoms or cyanobacteria amongst others
- Accumulate in a variety of filter feeding bivalves or shellfish and can reach high concentrations during algal blooms
  → "Harmful algal blooms" = HABs

# Introduction

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Massive Fish Kill in the Oder River in August 2022


https://www.tagesschau.de/ausland/fischsterben-oder-115.html

caused by the brackish haptophyte *Prymnesium parvum*

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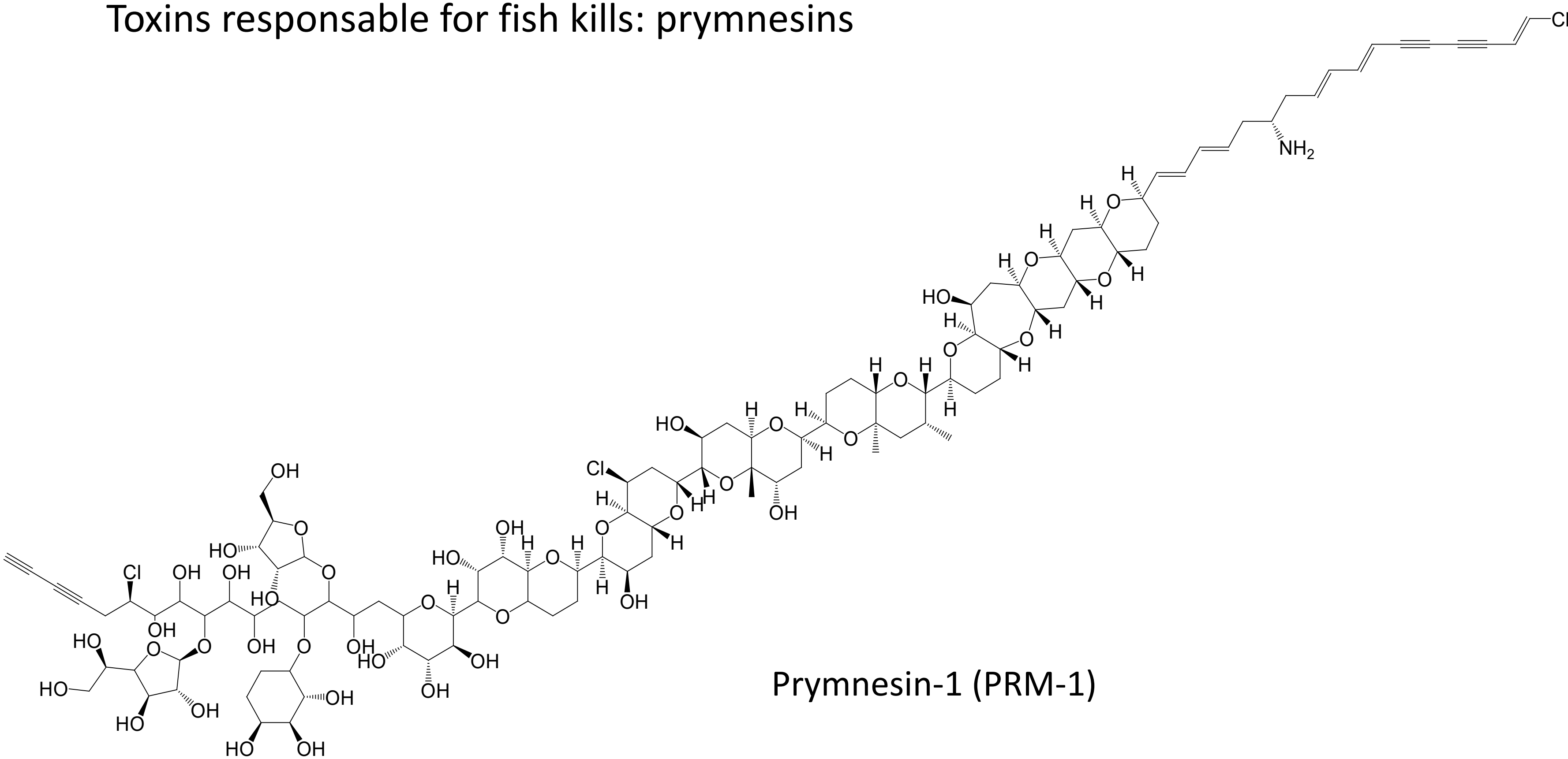
Toxins responsable for fish kills: prymnesins



Prymnesin-1 (PRM-1)

# Introduction

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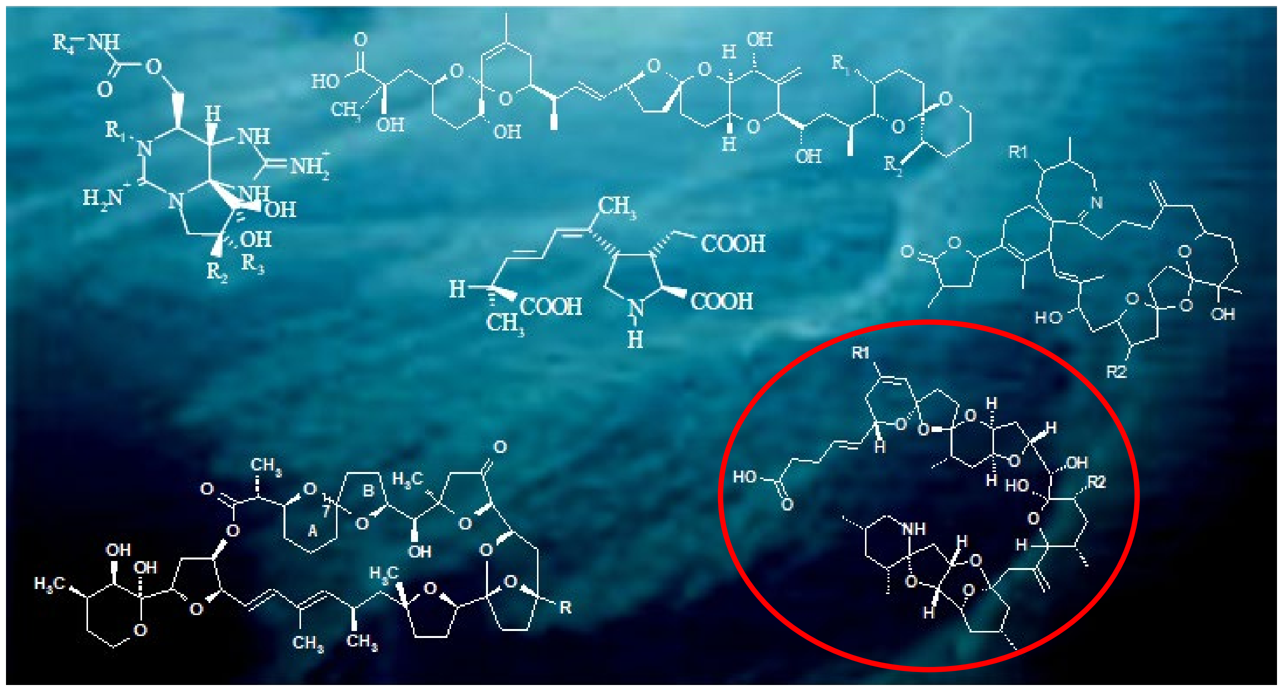
**Amphidinol**
**Anatoxin**
**Azaspiracid**
**Brevetoxin**
**Ciguatoxin**
**Cylindrospermopsin**
**Domoic Acid**
**Goniodomin**
**Gymnocin**
**Gymnodimine**
**Karenia brevisulcata toxin**
**Karlotoxin**
**Lyngbyatoxin**
**Microcystin**
**Nodularin**
**Okadaic acid**
**Palytoxin**
**Pectenotoxin**
**Pinnatoxin**
**Prymnesin**
**Saxitoxin**
**Spirolide**
**Yessotoxin**

## High variability of phycotoxin classes



Need of monitoring for seafood safety

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# Strategies for the Discovery of Source Organisms of Phycotoxins

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# 1) Biological approach

- Plankton sampling
- Species isolation
- Establishment of a monoclonal culture
- Toxin testing

# 2) Chemical approach

- Plankton sampling
- Toxin testing
- Species isolation
- Establishment of a monoclonal culture

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# Finding phycotoxin producers

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1995: 8 people in the Netherlands became ill after consumption of Irish mussels (*Mytilus edulis*) harvested at Killary Harbour (Ireland). Symptoms were like DSP intoxication, but DSP toxins were hardly present in the mussels (MacMahon & Silke, 1996: Harmful Algae News, 14, 2)

1998: Satake et al. identified azapiracid-1 (AZA-1) as the causative compound in shellfish (J. Am. Chem. Soc., 120, 9967-9968)

1999: Ofuji et al. elucidated the structures of AZA-2 and AZA-3 from shellfish (Nat. Toxins, 7, 99-102)

2001: Ofuji et al. elucidated the structures of AZA-4 and AZA-5 from shellfish (Biosci. Biotechnol. Biochem., 65, 470-472)

2003: James et al. elucidated the structures of AZA-6 to AZA-11 from shellfish (Toxicon, 41, 277-283)

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# Finding phycotoxin producers

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ELSEVIER

Toxicon 41 (2003) 145–151

**TOXICON**

www.elsevier.com/locate/toxicon

## Ubiquitous 'benign' alga emerges as the cause of shellfish contamination responsible for the human toxic syndrome, azaspiracid poisoning

Kevin J. James[a,*], Cian Moroney[a], Cilian Roden[a], Masayuki Satake[b], Takeshi Yasumoto[c], Mary Lehane[a], Ambrose Furey[a]

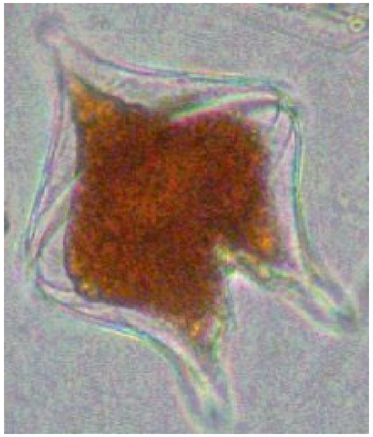
[a]*Proteobio, Department of Chemistry, Mass Spectrometry Centre for Proteomics and Biotoxin Research, Cork Institute of Technology, Bishopstown, Cork, Ireland*
[b]*Faculty of Agriculture, Tohoku University, Tsutsumidori-Amamiya, Aoba-ku, Sendai, Japan*
[c]*Japan Food Laboratories, Tama Laboratory, Nagayama, Tama-shi, Tokyo, Japan*

Received 5 June 2002; accepted 5 August 2002

**Abstract**

A new human toxic syndrome, azaspiracid poisoning (AZP), was identified following illness from the consumption of contaminated mussels (*Mytilus edulis*). To discover the aetiology of AZP, sensitive analytical protocols involving liquid chromatography–mass spectrometry (LC–MS) were used to screen marine phytoplankton for azaspiracids. Collections of single species were prepared by manually separating phytoplankton for LC–MS analysis. A dinoflagellate species of the genus, *Protoperidinium*, has been identified as the progenitor of azaspiracids. Azaspiracid-1, and its analogues, AZA2 and AZA3, were identified in extracts of 200 cells using electrospray multiple tandem MS. This discovery has significant implications for both human health and the aquaculture industry since this phytoplankton genus was previously considered to be toxicologically benign. The average toxin content was 1.8 fmol of total AZA toxins per cell with AZA1 as the predominant toxin, accounting for 82% of the total.


*Keywords:* Marine toxins; LC–MS; AZP; *Protoperidinium*; Shellfish poisoning



*Protoperidinium crassipes*

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# Finding phycotoxin producers

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ICMSS NEW ZEALAND 2007
6TH INTERNATIONAL CONFERENCE MOLLUSCAN SHELLFISH SAFETY

Correlations between the presence of known toxic phytoplankton species and toxin levels in shellfish in Irish waters 2002 – 2006

Siobhan Moran*, J Silke, C Cusack, P Hess
Marine Institute, Galway, Ireland
siobhan.moran@marine.ie

The Irish National Monitoring Programme for phytoplankton is part of the Irish Shellfish Biotoxin Monitoring Programme, which fulfills Regulation (EC) 853/2004. The four main toxic syndromes found in Irish waters are Diarrhetic, Paralytic, Amnesic, and Azaspiracid Shellfish Poisoning.

Over a four year period (2002 – 2006) there was no correlation between the occurrence of *Protoperidinium* spp. in plankton and azaspiracids in shellfish in Irish waters.

The authors exclude *Protoperidinium* as the source of azaspiracids

Possible reason for the misidentification of *Protoperidinium crassipes*:
*P. crassipes* as a heterotrophic dinoflagellate might have fed on the azaspiracid producing organism during a toxic event

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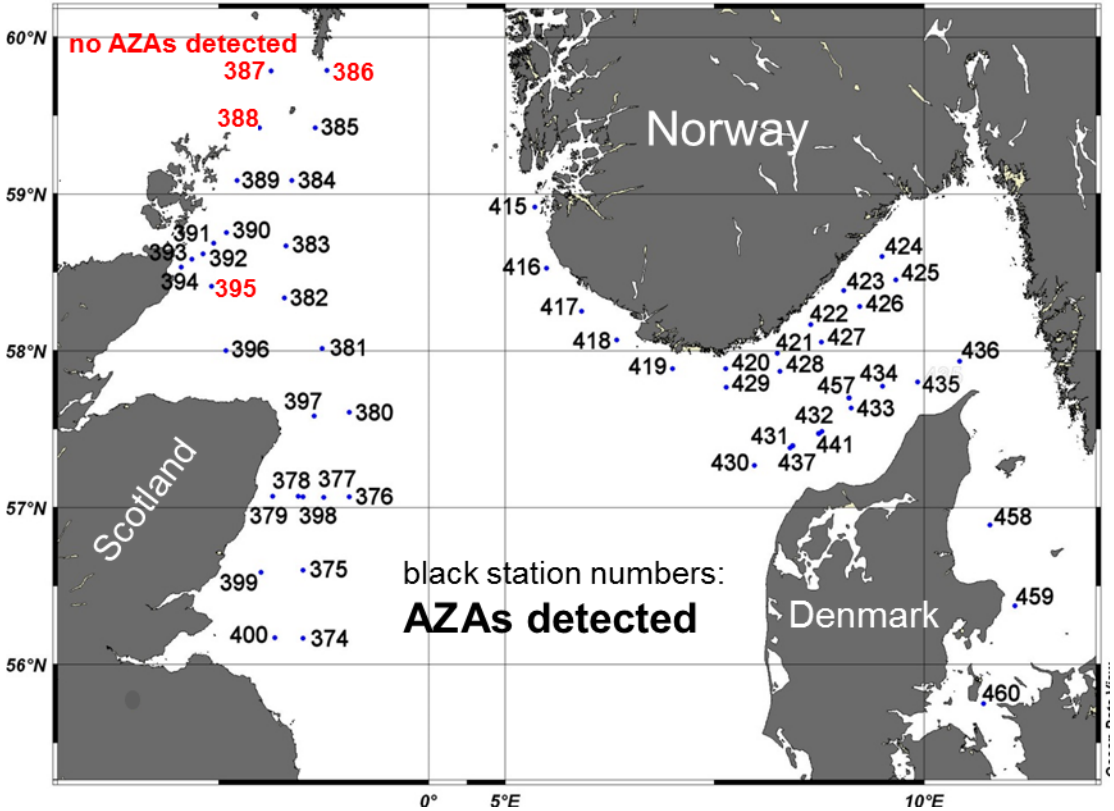
# Finding phycotoxin producers

Survey on the North Sea in July 2007



+





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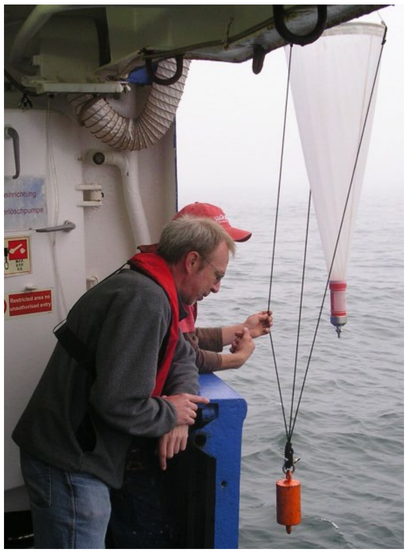
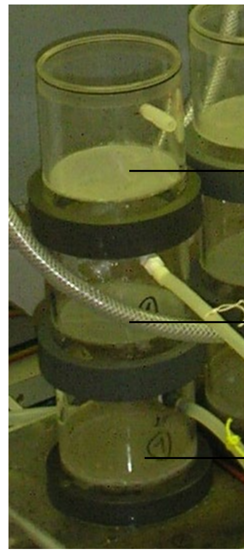
# Finding phycotoxin producers



Plankton net
pore size 20 µm



Filter array

200 µm (zooplankton)

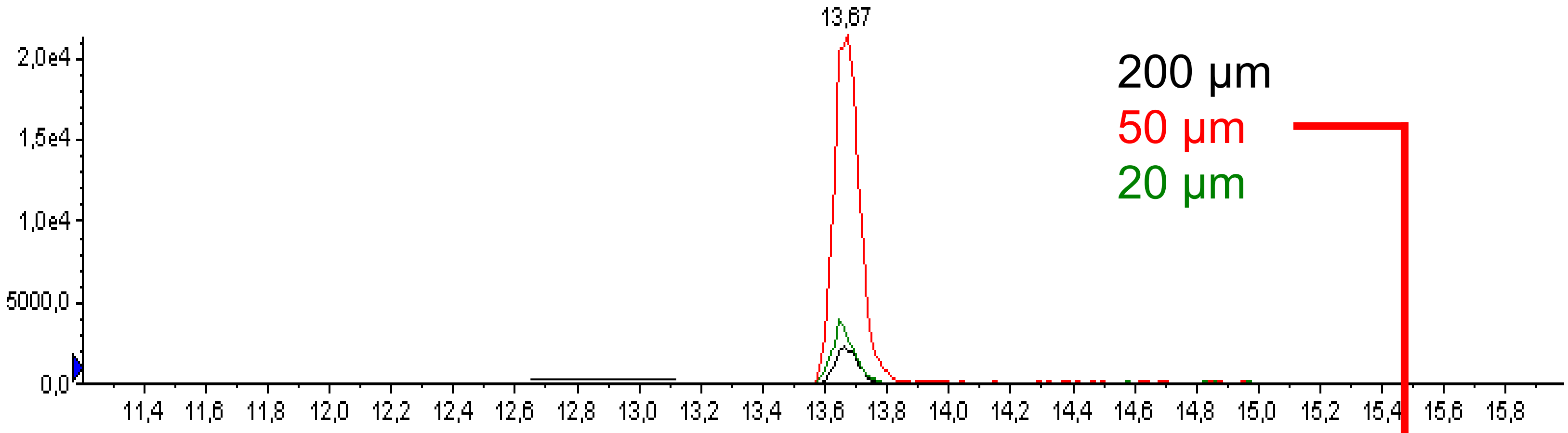
50 µm (big phytoplankton)

20 µm (small phytoplankton)

LC-MS/MS

# Finding phycotoxin producers

Size fraction test



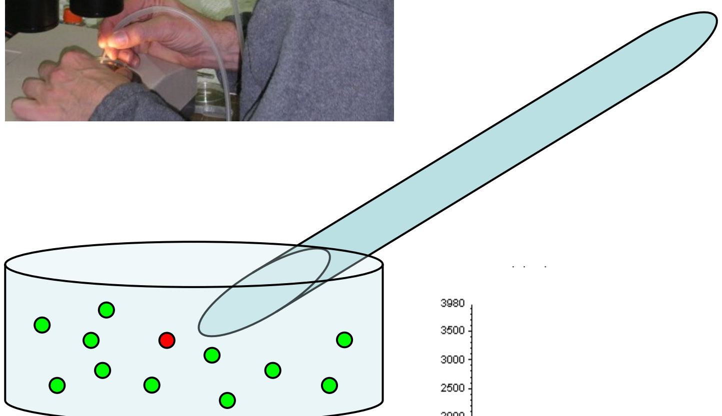

Look into 50 µm plankton fraction

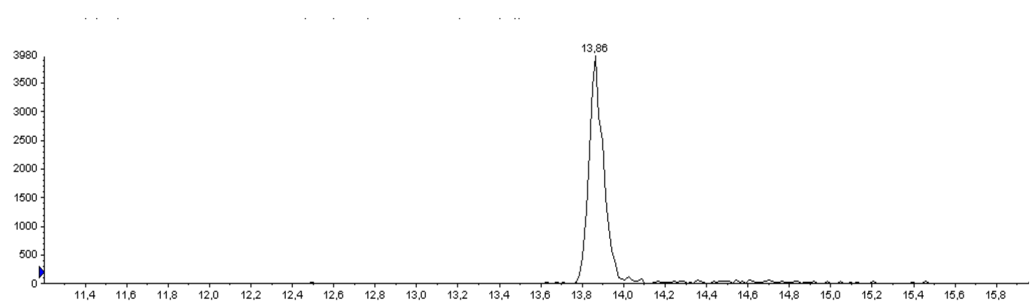

*Favella ehrenbergii*

# Finding phycotoxin producers

Single cell isolation of 160 *F. ehrenbergii* individuals with a microcapillary

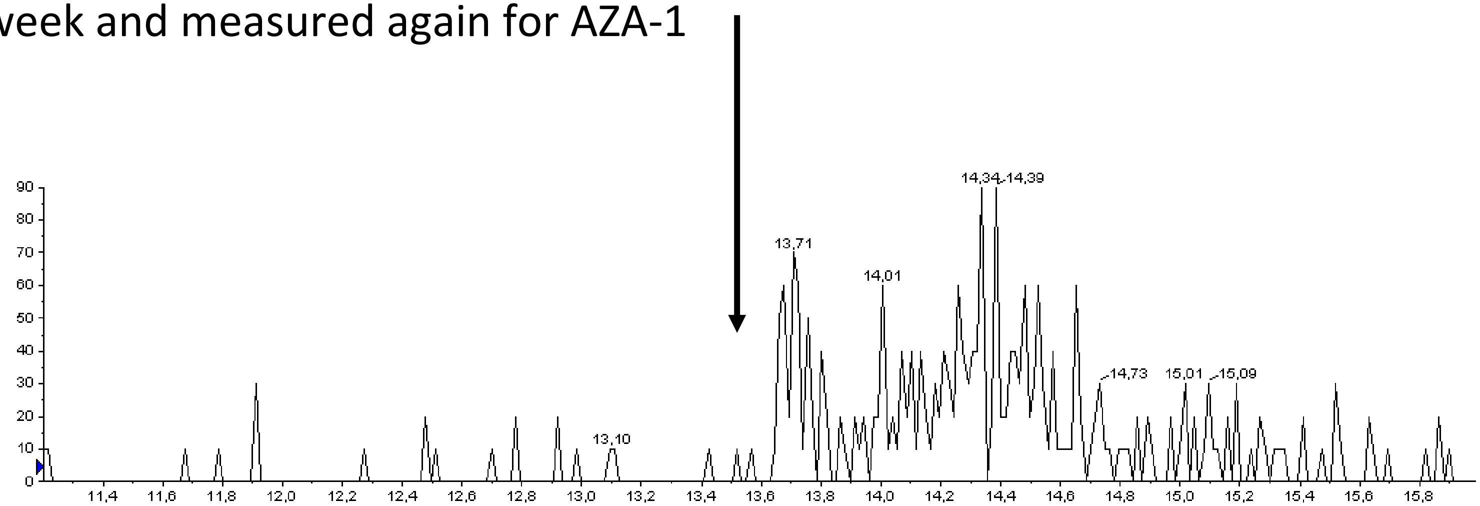
Liquid chromatography-mass spectrometry (LC/MS)





# Finding phycotoxin producers

Azaspiracid containing *F. ehrenbergii* were fed with non-toxic *Scrippsiella* for one week and measured again for AZA-1




Liquid chromatography-mass spectrometry (LC/MS)

*Favella ehrenbergii*

=> *F. ehrenbergii* is not an azaspiracid producer!

# Finding phycotoxin producers

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If *Favella* feeds on
AZA producers,
they cannot be very
big!

**=> screening of size fractions < 20 µm for AZA**

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Screening of plankton < 20 µm for AZA

Rosette sampler
(unfiltered water samples)

Filtration over 20 µm mesh

Removal of plankton > 20 µm

Filtration over 10 µm (mesh), 8 µm, 3 µm and 0.2 µm (polycarbonate filters)

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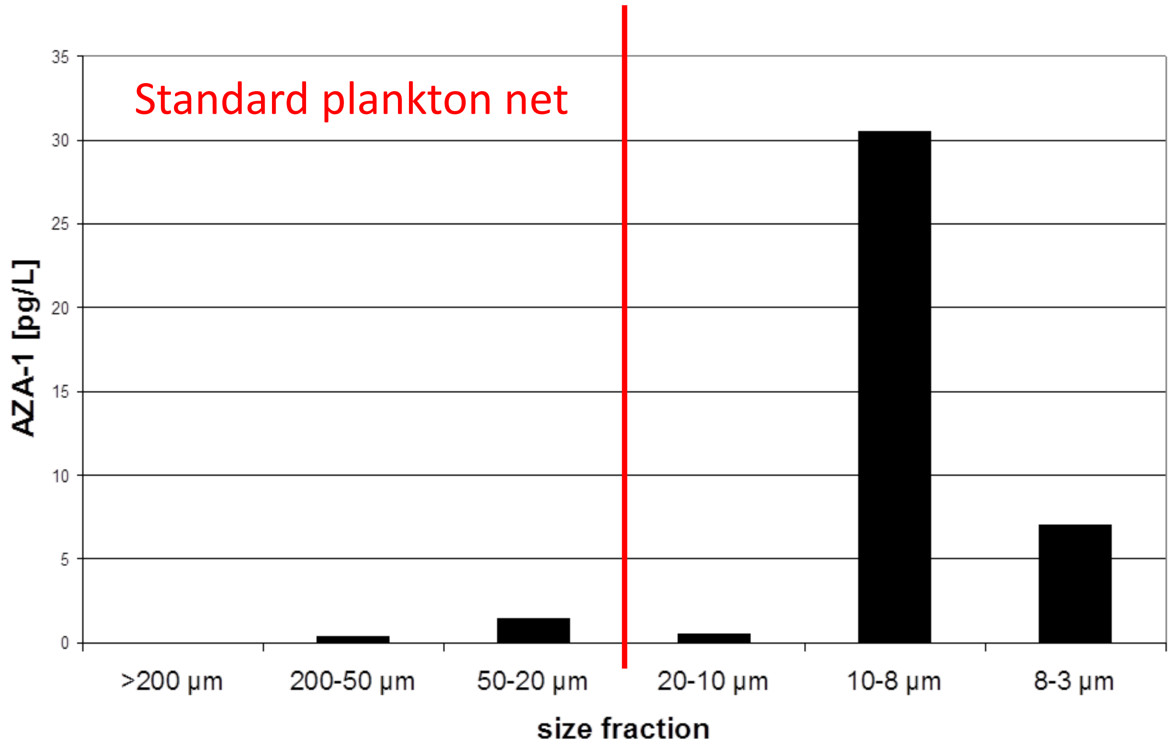
# Finding phycotoxin producers




=> the AZA producer is smaller than 10 µm

=> the AZA producer can only be sampled by direct water collection, but not by phytoplankton net tows

# Finding phycotoxin producers

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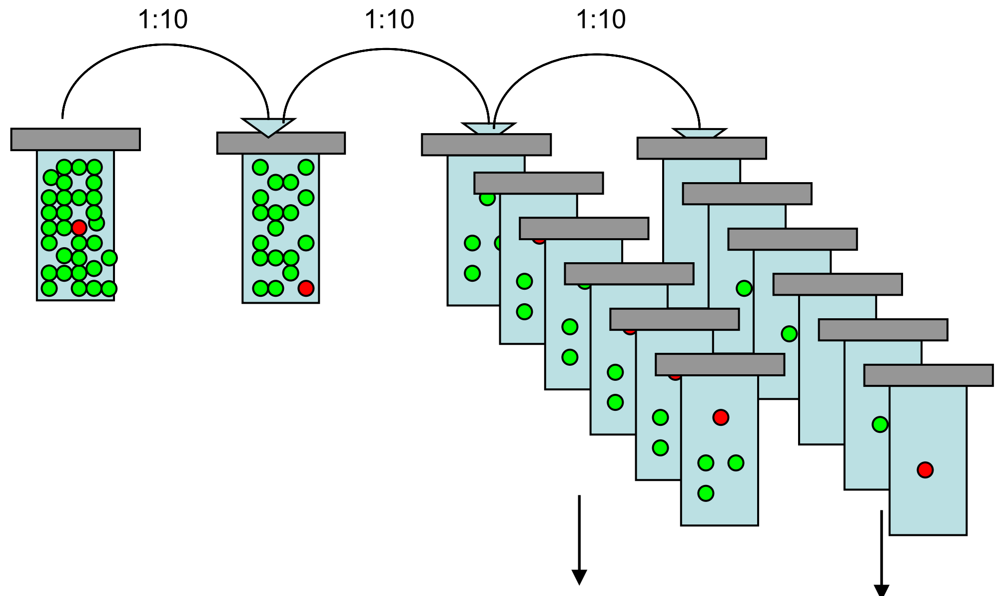
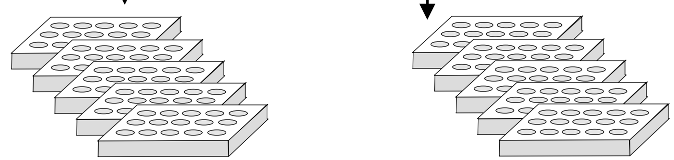
AZA containing plankton sample form the field

Serial dilution method




Incubation and growth
in multiwell-plates



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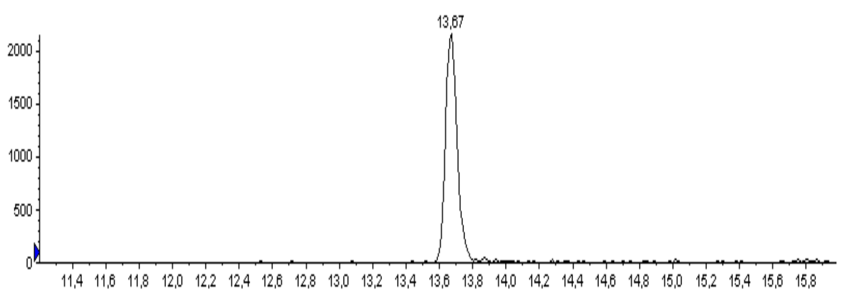
# Finding phycotoxin producers

Out of 240 isolates tested

only one culture “3 D9“
contained AZA-1

AZA-1





Krock, B., et al., 2009, Harmful Algae 8(2): 254-263.

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# Elucidation of the High Structural Variability of Phycotoxins in Plankton and Higher Trophic Levels of the Food Chain

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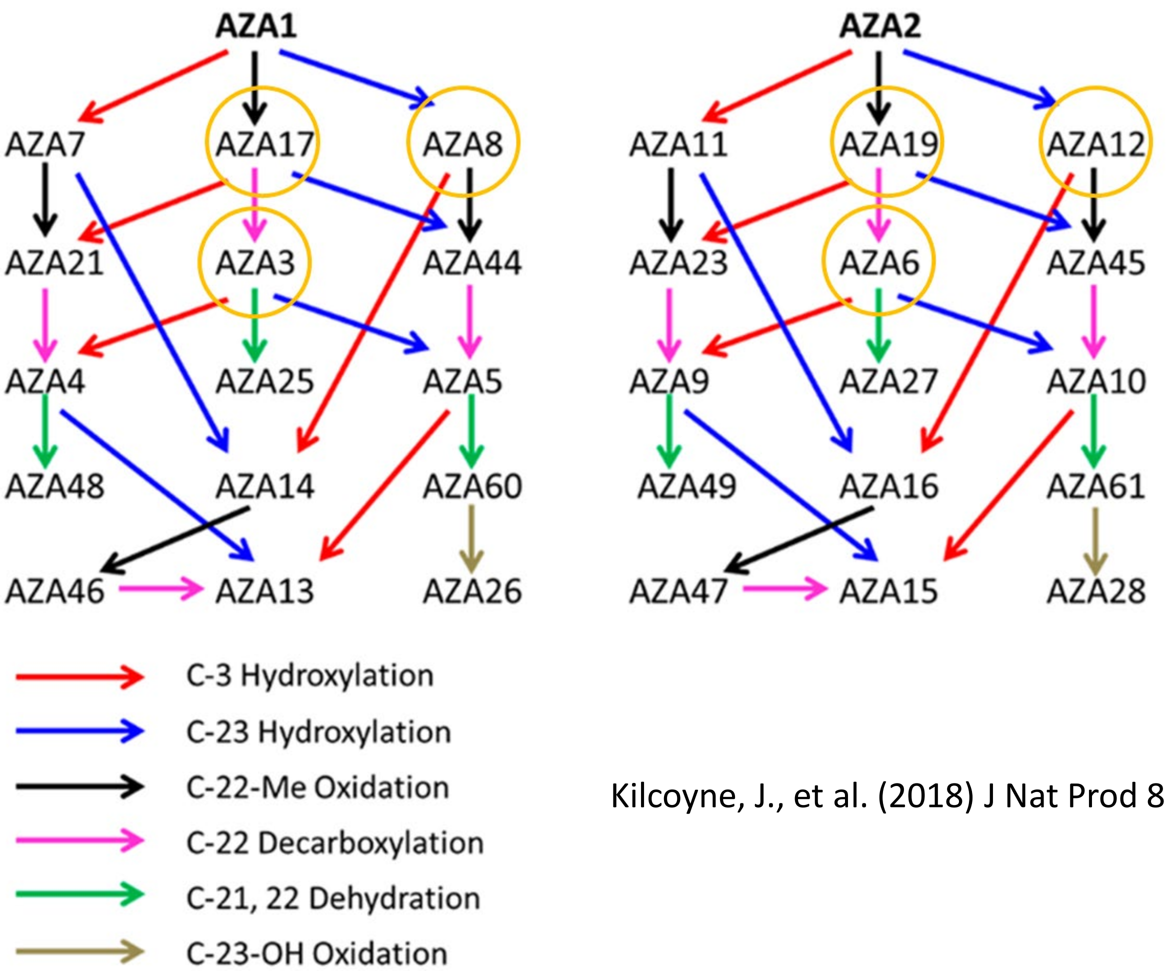
# Structural variability of phycotoxins

## Azaspiracids – Metabolism in Bivalves




Kilcoyne, J., et al. (2018) J Nat Prod 81(4), 885-893.

# Structural variability of phycotoxins

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| | |
|---|---|
| AZA-1 | AZA-41 |
| AZA-2 | AZA-42 |
| epi-AZA-7 | AZA-43 |
| AZA-11 | AZA-50 |
| AZA-33 | AZA-51 |
| AZA-34 | AZA-52 |
| AZA-35 | AZA-53 |
| AZA-36 | AZA-54 |
| AZA-37 | AZA-55 |
| AZA-38 | AZA-56 |
| AZA-39 | AZA-57 |
| AZA-40 | AZA-58 |
| AZA-59 | AZA-62 |

| | | | |
|---|---|---|---|
| AZA-3 | AZA-14 | AZA-25 | AZA-47 |
| AZA-4 | AZA-15 | AZA-26 | AZA-48 |
| AZA-5[+] | AZA-16 | AZA-27 | AZA-49 |
| AZA-6 | AZA-17 | AZA-28 | AZA-60 |
| AZA-7 | AZA-18 | AZA-29 | AZA-61 |
| AZA-8 | AZA-19 | AZA-30 | |
| AZA-9 | AZA-20 | AZA-31 | |
| AZA-10 | AZA-21 | AZA-32 | |
| AZA-11* | AZA-22 | AZA-44 | |
| AZA-12 | AZA-23 | AZA-45 | |
| AZA-13 | AZA-24 | AZA-46 | |

* Tillmann, U., et al. (2017) J Plankt Res 39(2), 350-367.
[+] Montoya, N., pers. commun.

Krock, B., et al. (2019) Harmful Algae 82, 1-8.
[1] Additional planktonic AZA reported by Ozawa, M., et al. (2021) Toxicon 199: 145-155.

**Two AZAs of phytoplankton origin result in 38 shellfish metabolites!**

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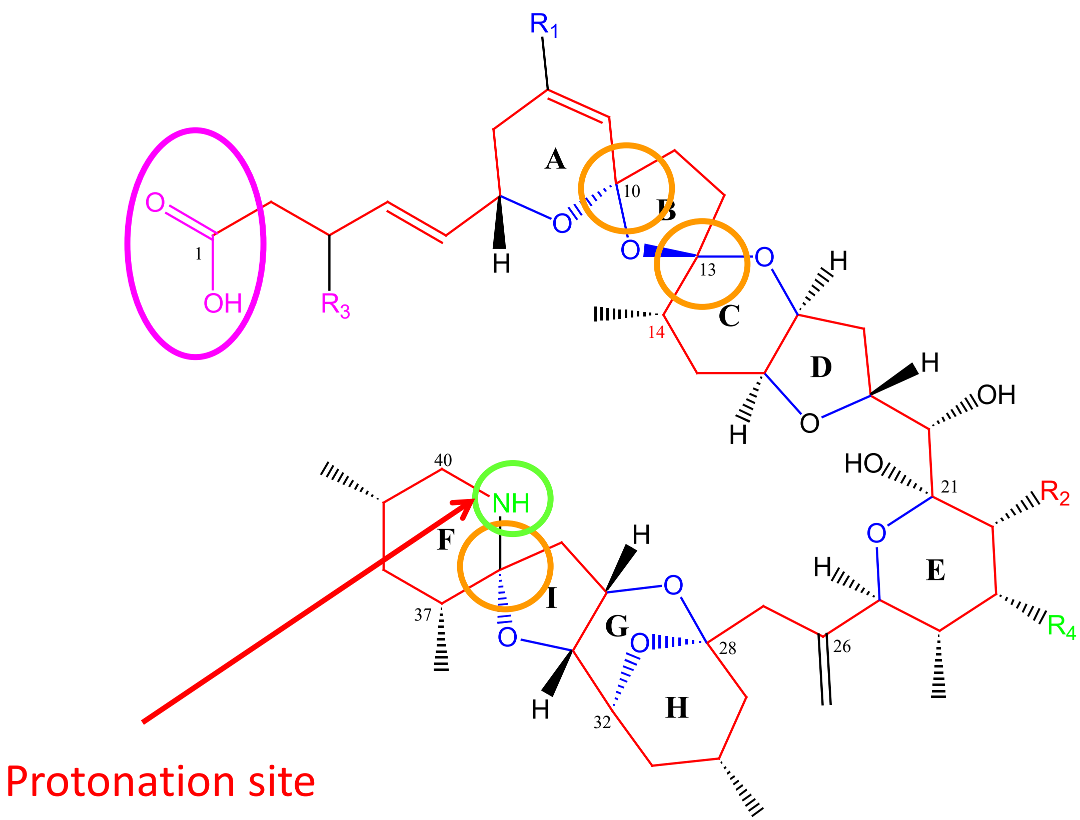
# Structural variability of phycotoxins




azaspiracid

Polyketide:
linear carbon skeleton
with cyclic ether bridges

amino function
chemical nomenclature:
aza = secondary amine

spiro function

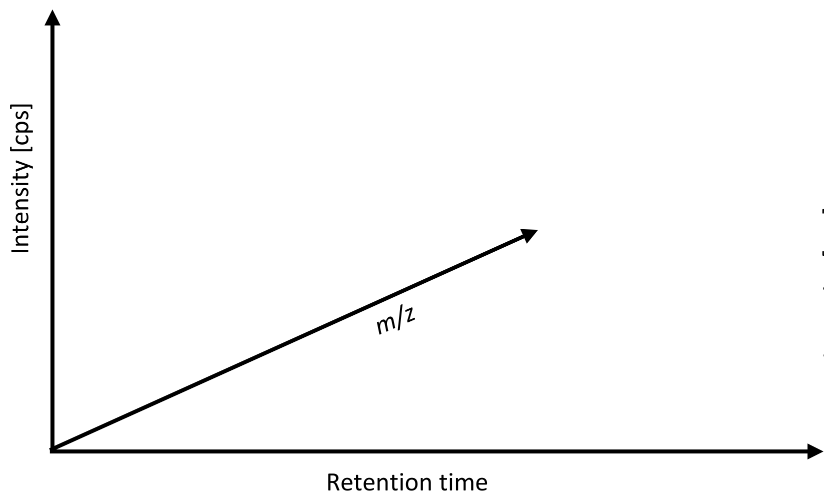
acid

# Structural variability of phycotoxins

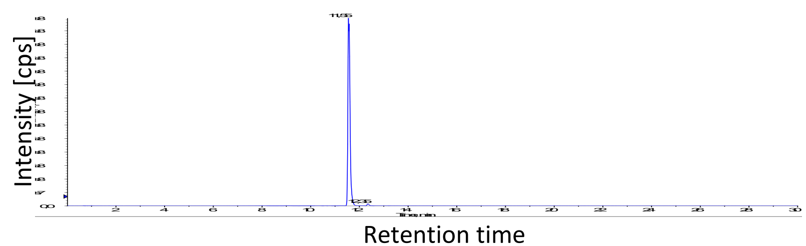
## LC-MS/MS data set

LC-MS data are a three dimensional data set:

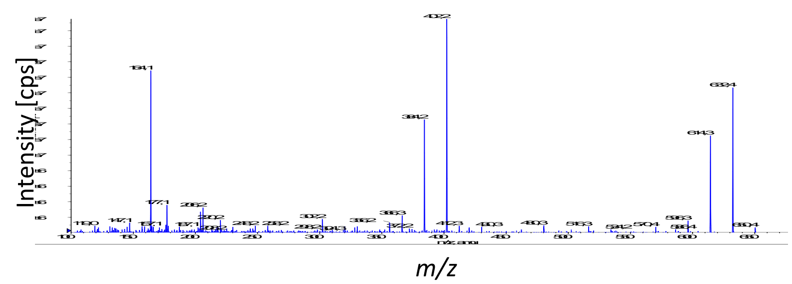
1. Retention time (chromatography)
2. Mass axis *(m/z)* (mass spectrometry)
3. Ion intensity



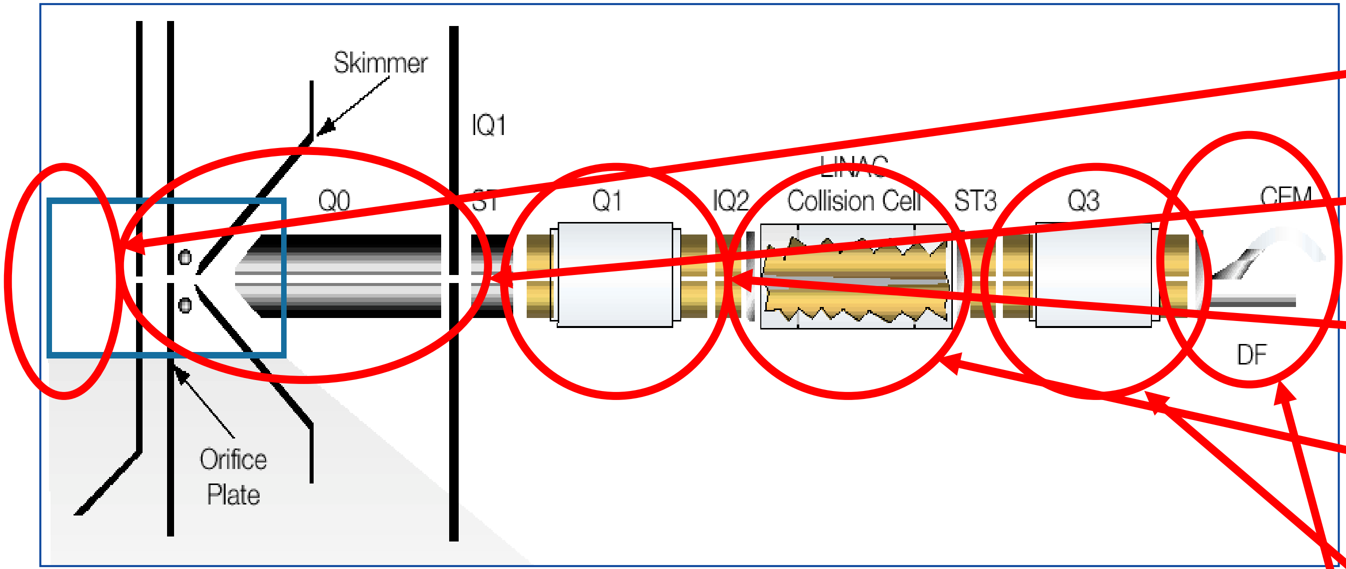

LC-MS chromatogram




LC-MS spectrum



Scheme of a Triple Quadrupole Instrument




Ion production

↓

Ion transport

↓

Ion filtering

↓

Ion processing

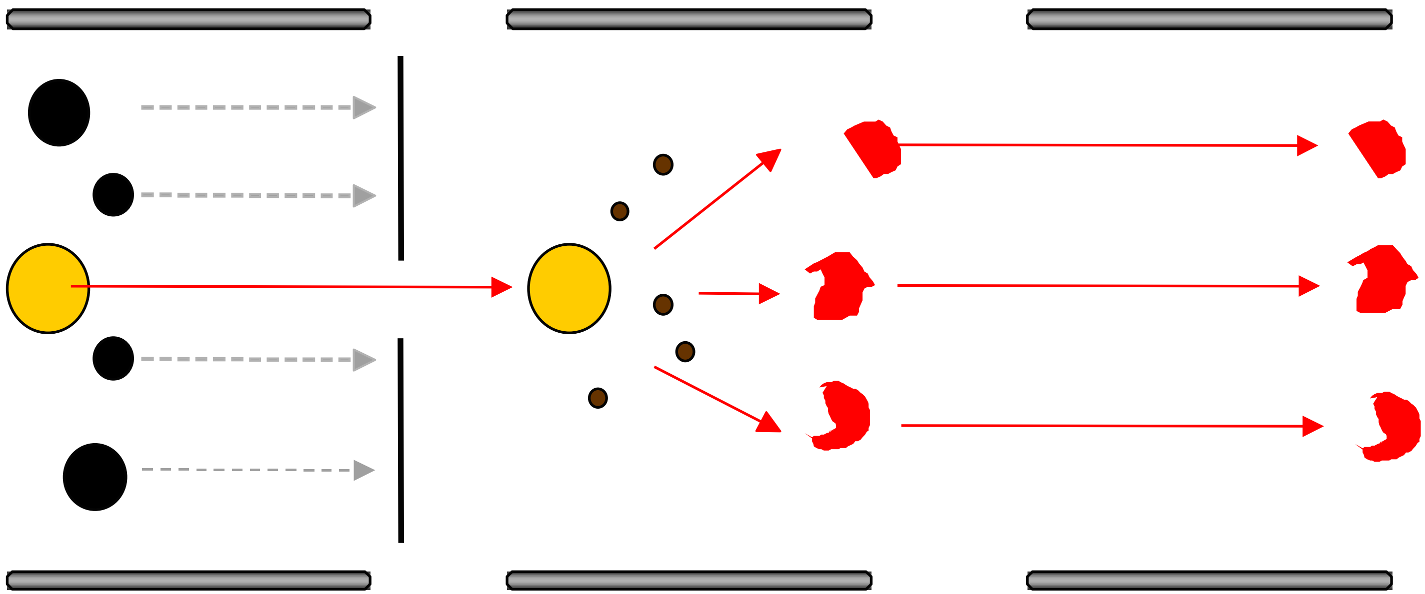
↓

Ion filtering

↓

Ion detection

## Product (Daughter) Ion Scan

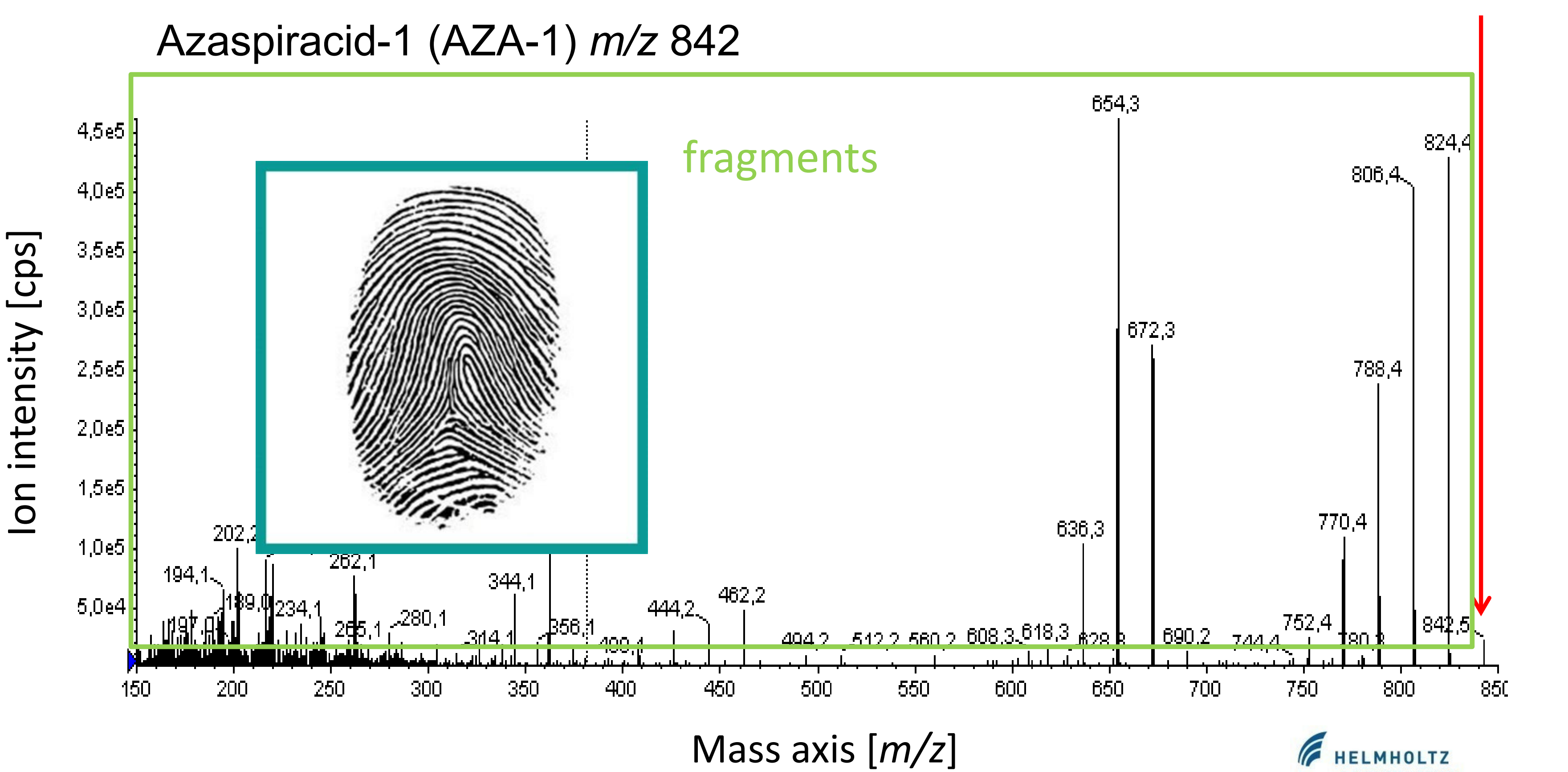


Q1 | Q2 | Q3

mass filter | collision cell fragmentation | scan

# Structural variability of phycotoxins

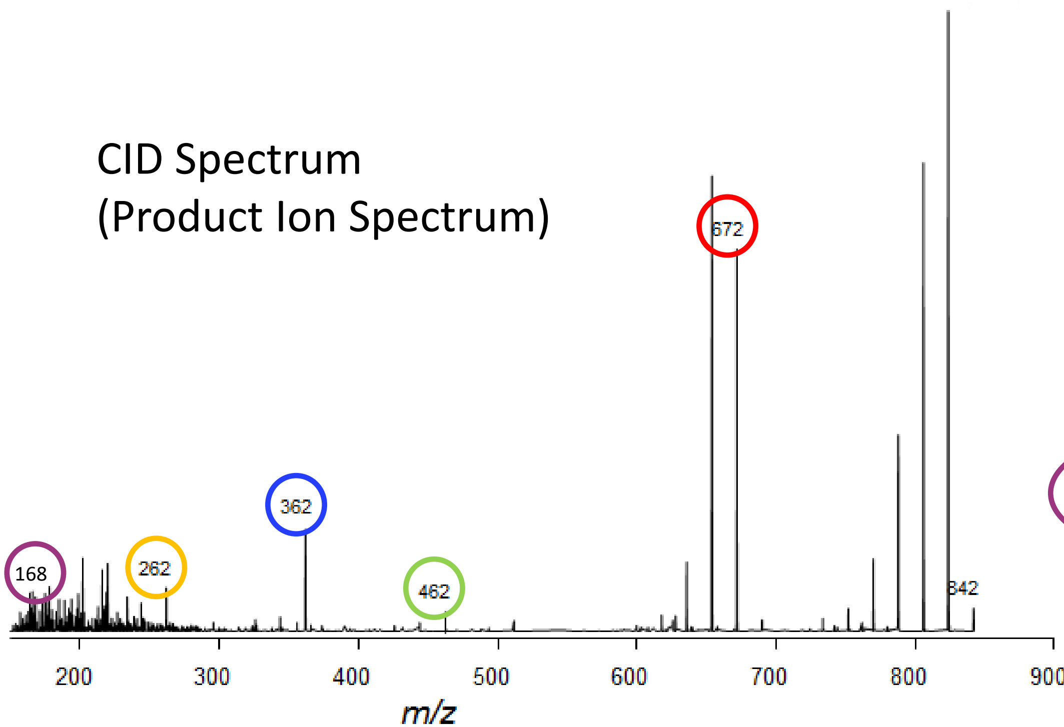
Collision induced dissociation (CID) spectrum
Product ion spectrum
Mass spectrum

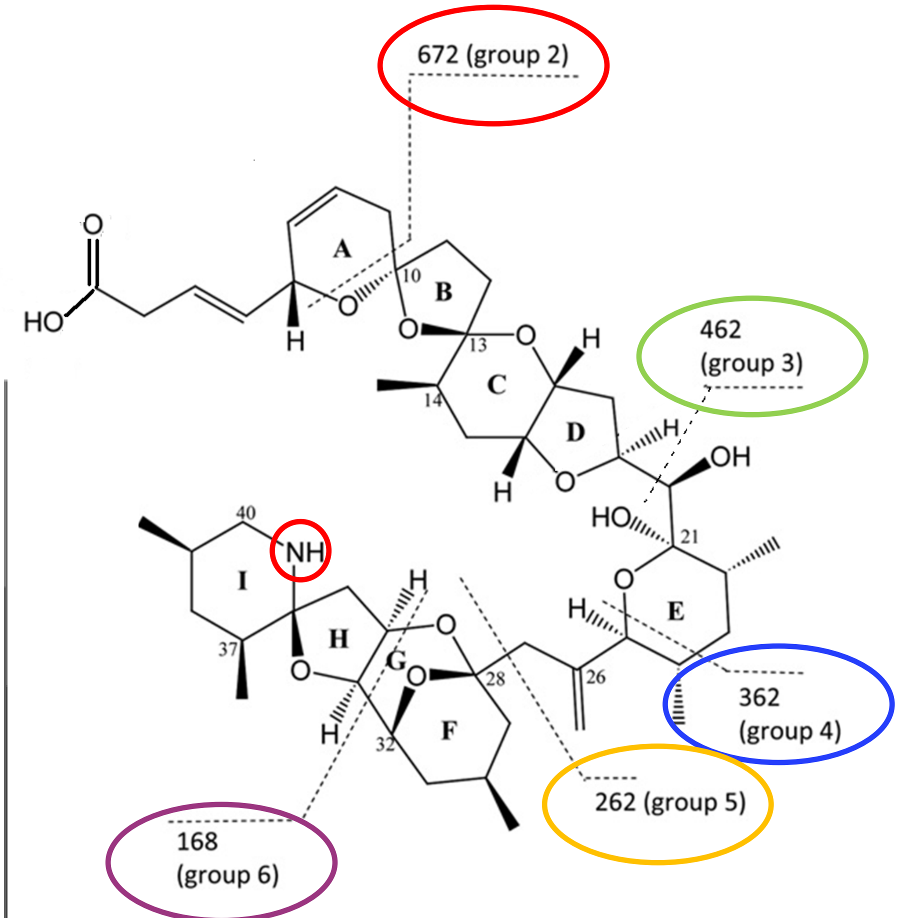


# Structural variability of phycotoxins

Azaspiracid-1 (AZA-1) *m/z* 842

CID Spectrum
(Product Ion Spectrum)

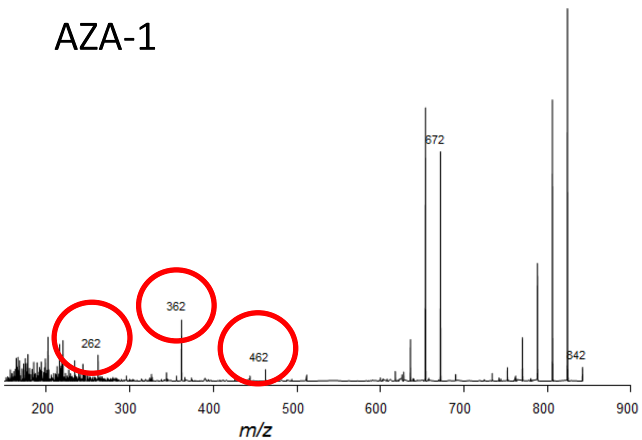


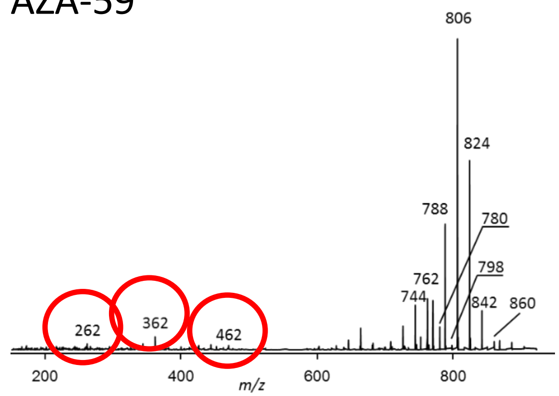
# Structural variability of phycotoxins

## Product Ion (CID) Spectra of Azaspiracids

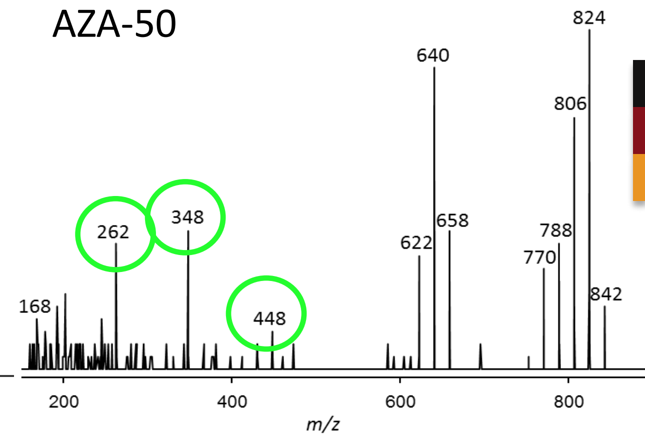
AZA-1



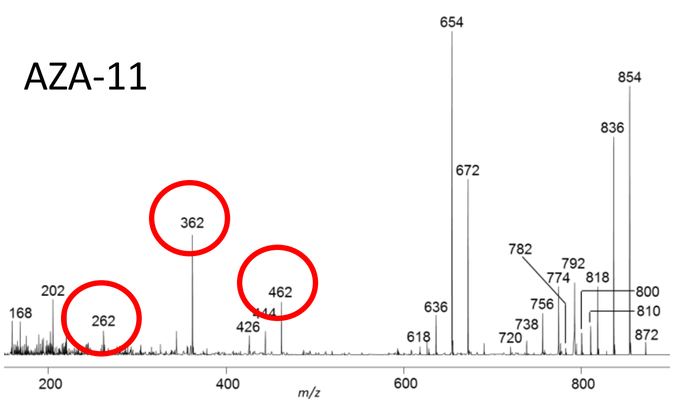

AZA-59



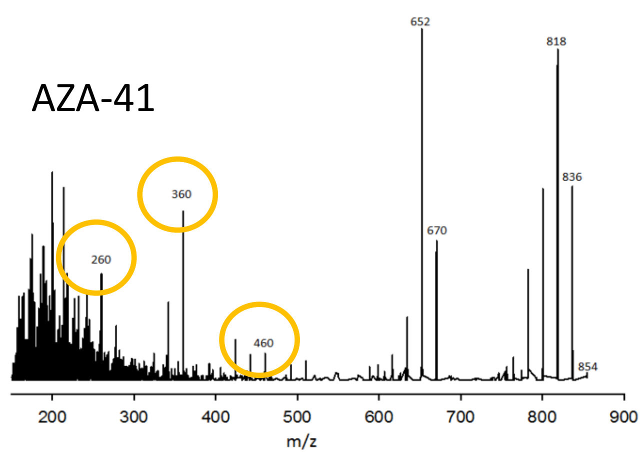

AZA-50



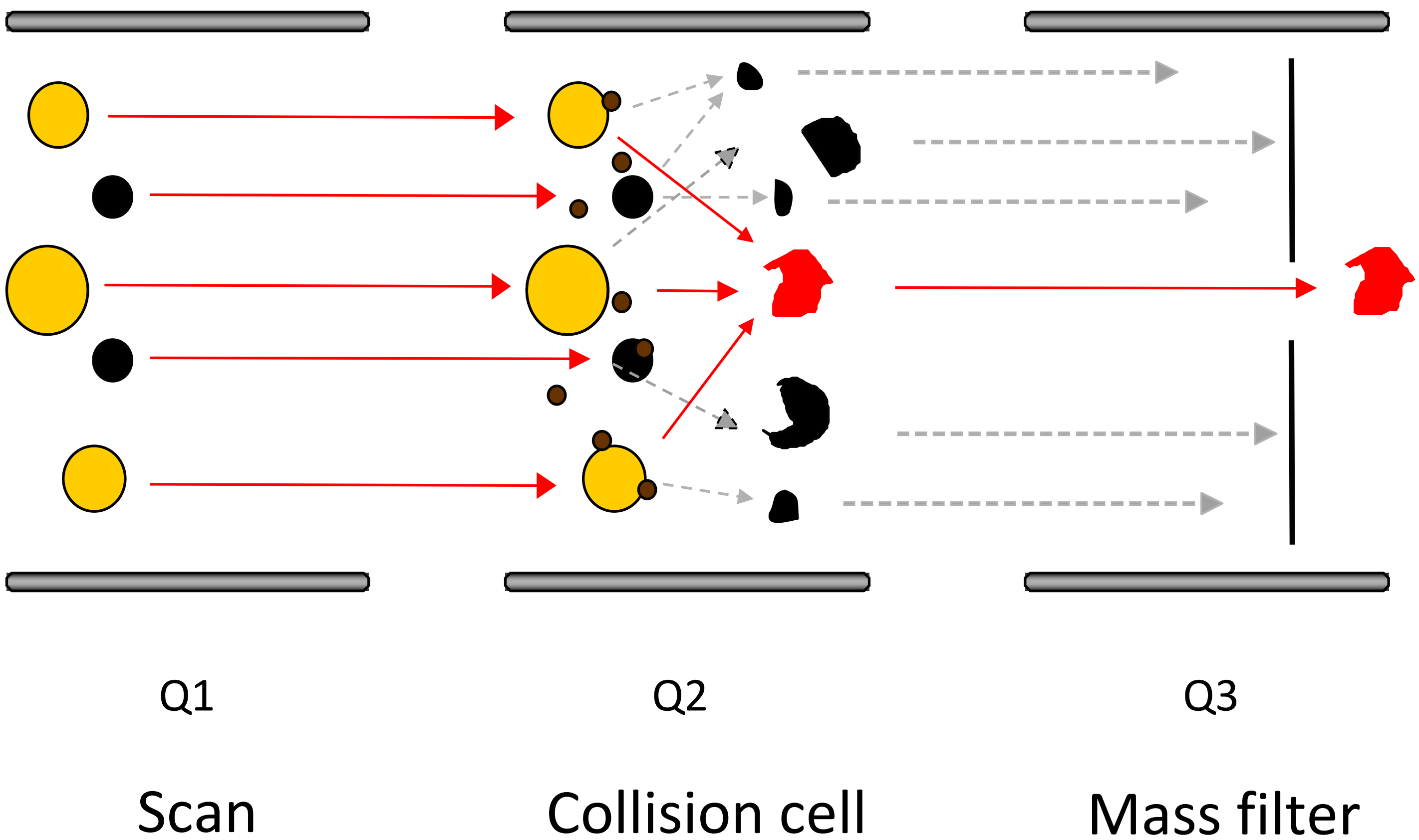

AZA-11



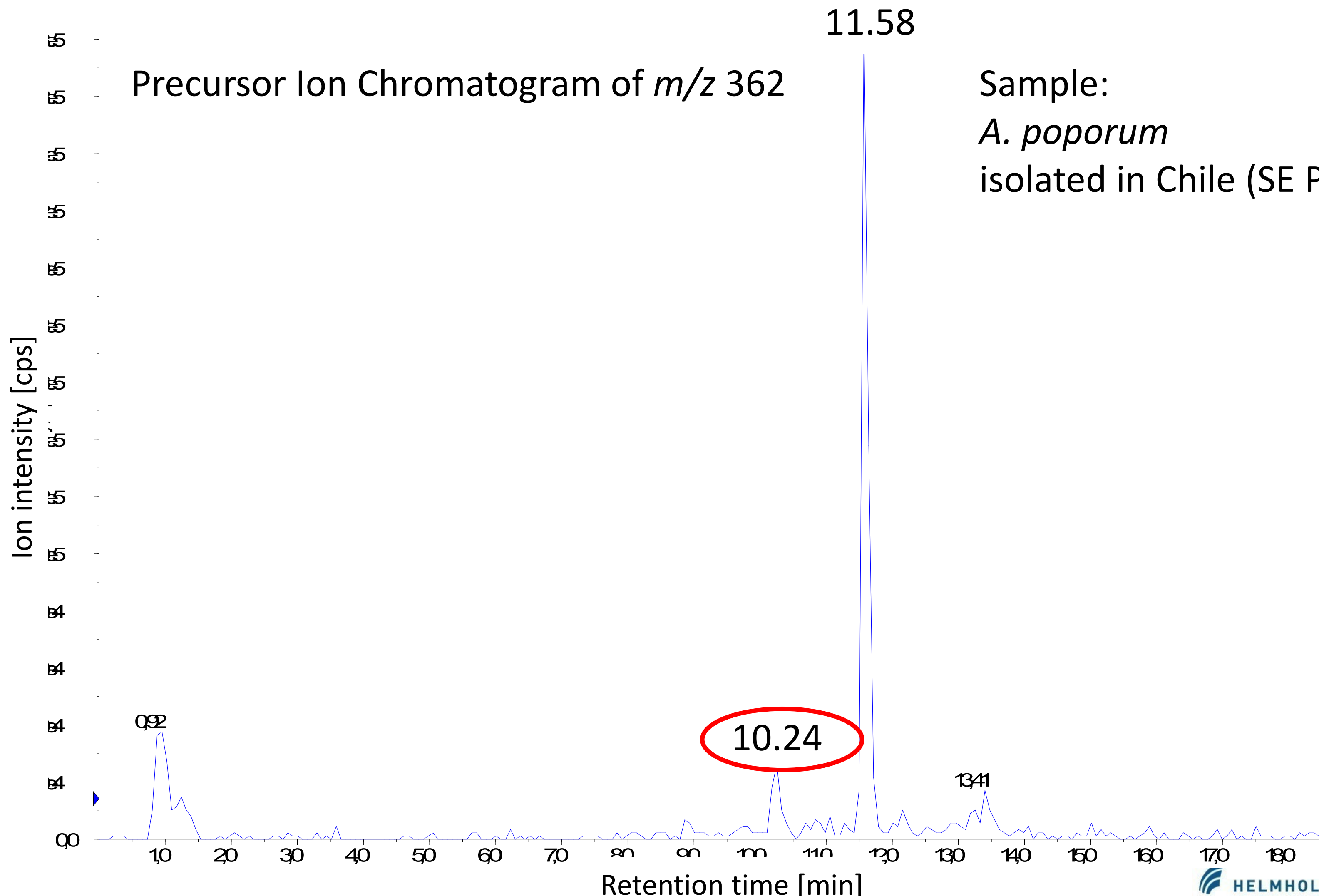

AZA-41



# Search for Unknowns – Precursor Scan



# Structural variability of phycotoxins



# Structural variability of phycotoxins

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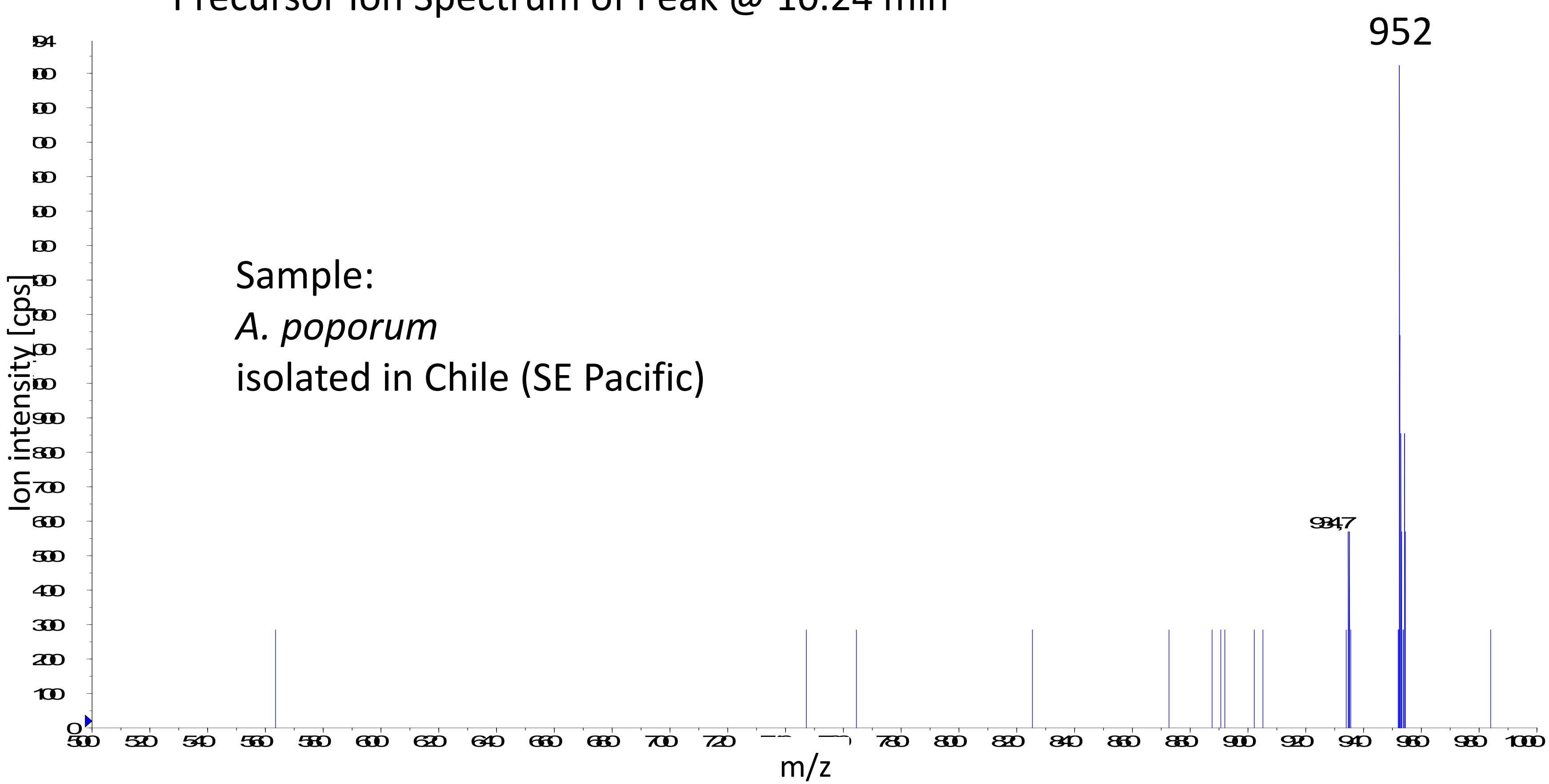
Precursor Ion Spectrum of Peak @ 10.24 min

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# Structural variability of phycotoxins

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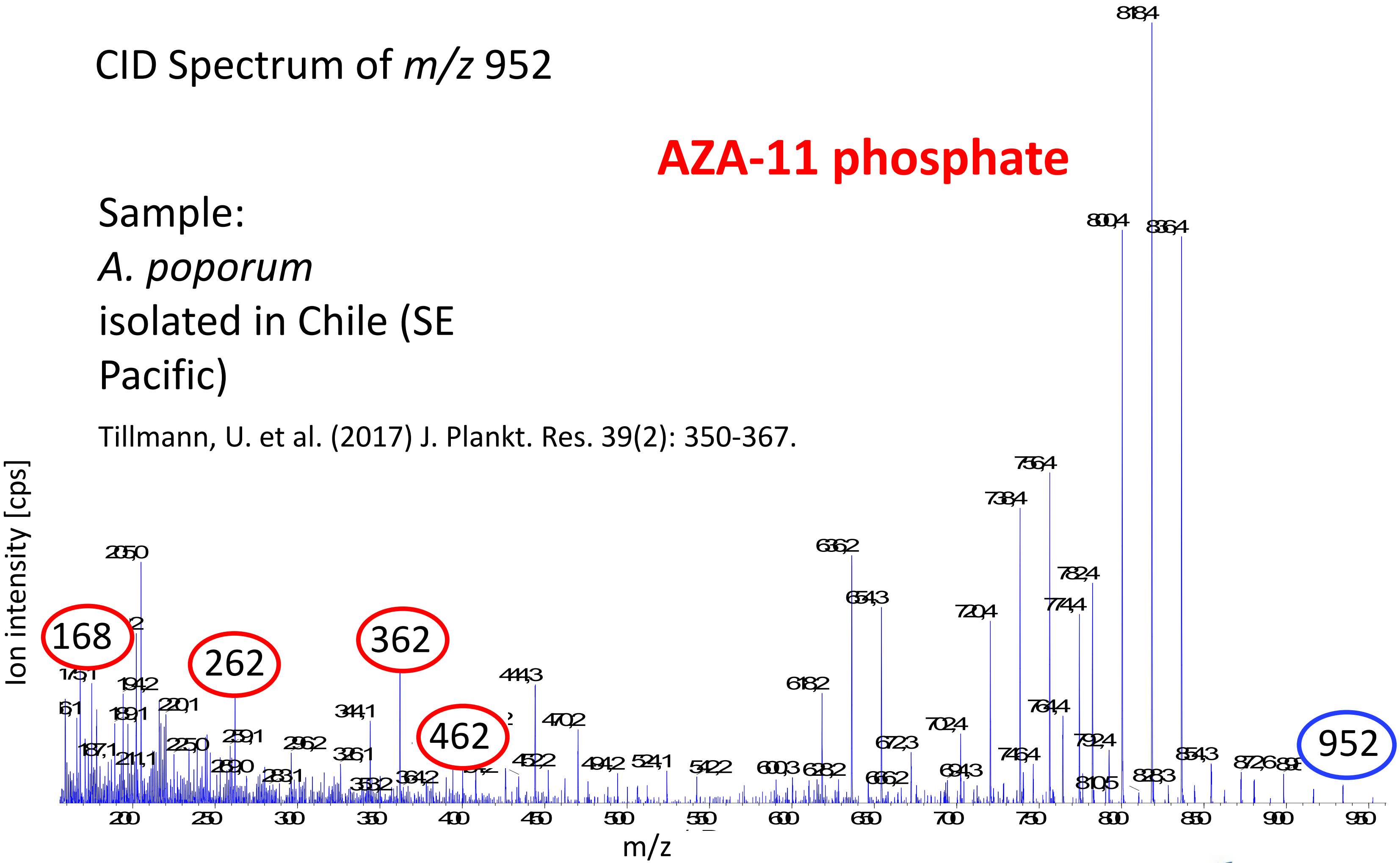
CID Spectrum of *m/z* 952

Sample:
*A. poporum*
isolated in Chile (SE Pacific)

Tillmann, U. et al. (2017) J. Plankt. Res. 39(2): 350-367.

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# Occurrence and Spatial Distribution of Phycotoxins and Toxigenic Microalgal Species

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# Occurrence of Toxins and their Producers

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Currently Known AZA from Dinoflagellate (Algal) Origin

| # | AZA | *m/z* $[M+H]^+$ | *m/z* group 4 fragment | *m/z* group 5 fragment | Producer | Reference |
|---|---|---|---|---|---|---|
| 1 | AZA-1 | 842 | 362 | 262 | *A. spinosum* | Krock et al. 2009 |
| 2 | AZA-2 | 856 | 362 | 262 | *A. spinosum*<br>*A. poporum*<br>*Am. languida* | Krock et al. 2009<br>Krock et al. 2014<br>Tillmann et al. 2017 |
| 3 | epi-AZ[illegible] | [illegible] | [illegible] | [illegible] | *A. dexteroporum* | Rossi et al. 2017 |
| 4 | AZA-1[illegible] | [illegible] | [illegible] | [illegible] | *A. poporum* | Krock et al. 2014 |
| 5 | AZA-3[illegible] | [illegible] | [illegible] | [illegible] | *A. spinosum* | Kilcoyne et al. 2014 |
| 6 | AZA-3[illegible] | [illegible] | [illegible] | [illegible] | *A. spinosum* | {Kilcoyne et al. 2014 |
| 7 | AZA-35 | 830 | 362 | 262 | *A. spinosum*<br>*A. dexteroporum* | Kilcoyne et al. 2014<br>Rossi et al. 2017 |
| 8 | AZA-3[illegible] | [illegible] | [illegible] | [illegible] | *A. poporum* | Krock et al. 2015 |
| 9 | AZA-3[illegible] | [illegible] | [illegible] | [illegible] | *A. poporum* | Krock et al. 2015 |
| 10 | AZA-3[illegible] | [illegible] | [illegible] | [illegible] | *Am. languida* | Krock et al. 2012 |
| 11 | AZA-3[illegible] | [illegible] | [illegible] | [illegible] | *Am. languida* | Krock et al. 2012 |
| 12 | AZA-4[illegible] | [illegible] | [illegible] | [illegible] | *A. poporum* | Krock et al. 2014 |
| 13 | AZA-41 | [illegible] | [illegible] | [illegible] | *A. poporum* | Krock et al. 2014 |
| 14 | AZA-42 | 870 | 360 | 260 | *A. poporum* | Krock et al. under review |
| 15 | AZA-43 | 828 | 360 | 260 | *Am. languida* | Tillmann et al. 2017 |
| 16 | AZA-5[illegible] | [illegible] | [illegible] | [illegible] | *A. spinosum* | Tillmann et al. 2018 |
| 17 | AZA-5[illegible] | [illegible] | [illegible] | [illegible] | *A. spinosum* | Tillmann et al. 2018 |
| 18 | AZA-5[illegible] | [illegible] | [illegible] | [illegible] | *Am. languida* | Tillmann et al. 2018 |
| 19 | AZA-5[illegible] | [illegible] | [illegible] | [illegible] | *Am. languida* | Tillmann et al. 2018 |
| 20 | AZA-5[illegible] | [illegible] | [illegible] | [illegible] | *A. dexteroporum* | Rossi et al. 2017 |
| 21 | AZA-5[illegible] | [illegible] | [illegible] | [illegible] | *A. dexteroporum* | Rossi et al. 2017 |
| 22 | AZA-5[illegible] | [illegible] | [illegible] | [illegible] | *A. dexteroporum* | Rossi et al. 2017 |
| 23 | AZA-57 | 844 | 362 | 262 | *A. dexteroporum* | Rossi et al. 2017 |
| 24 | AZA-58 | 828 | 362 | 262 | *A. dexteroporum* | Rossi et al. 2017 |
| 25 | AZA-59 | 860 | 362 | 262 | *A. poporum* | Kim et al. 2017 |
| 26 | AZA-62 | 870 | 362 | 262 | *A. poporum* | Krock et al. 2019 |

26 AZAs from planktonic origin

Currently 62 published AZAs

And at least additional 10 known AZAs

Krock, B., et al. 2019 Harmful Algae 82: 1-8.

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# Occurrence of Toxins and their Producers

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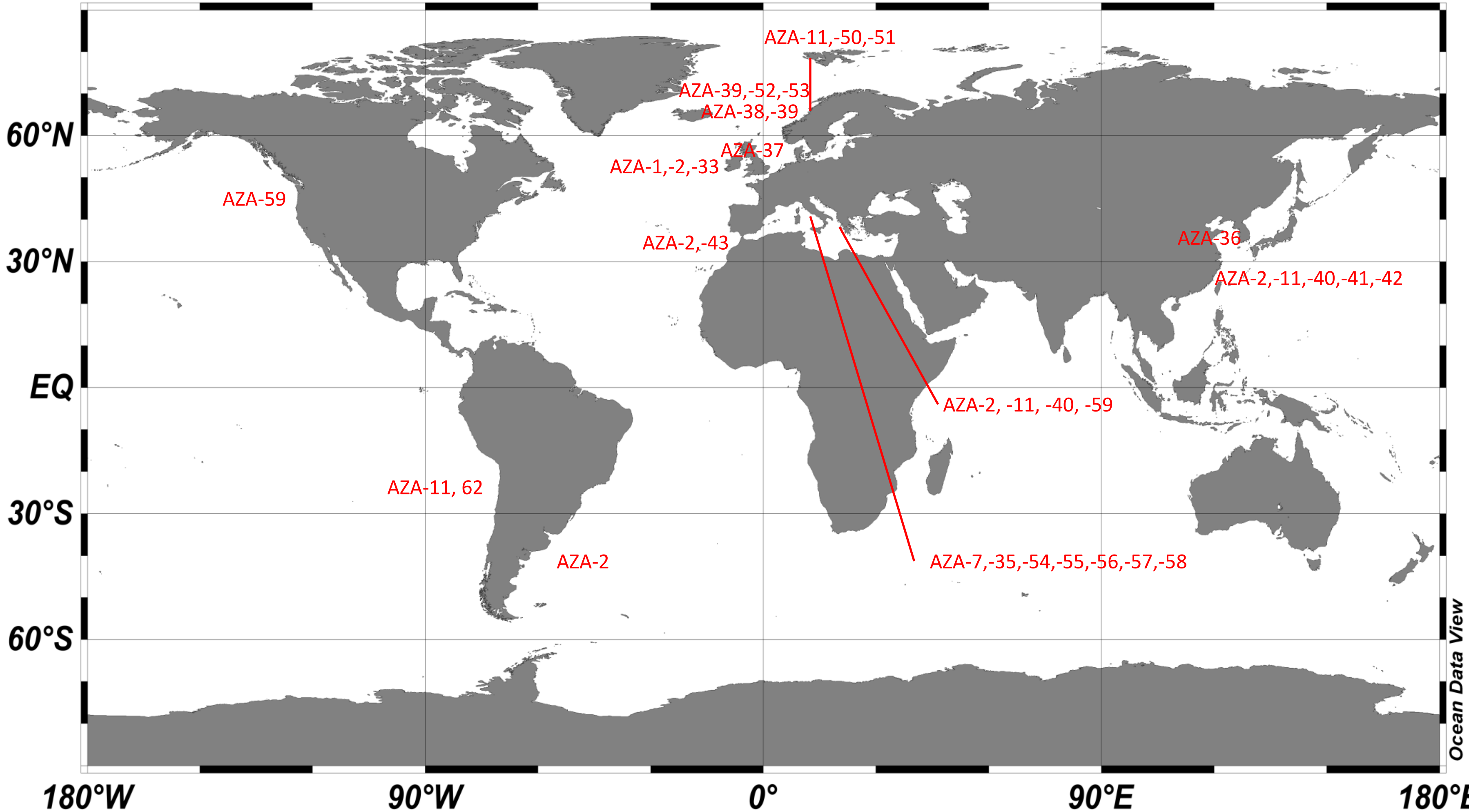
## Azaspiracids – Geographic distribution



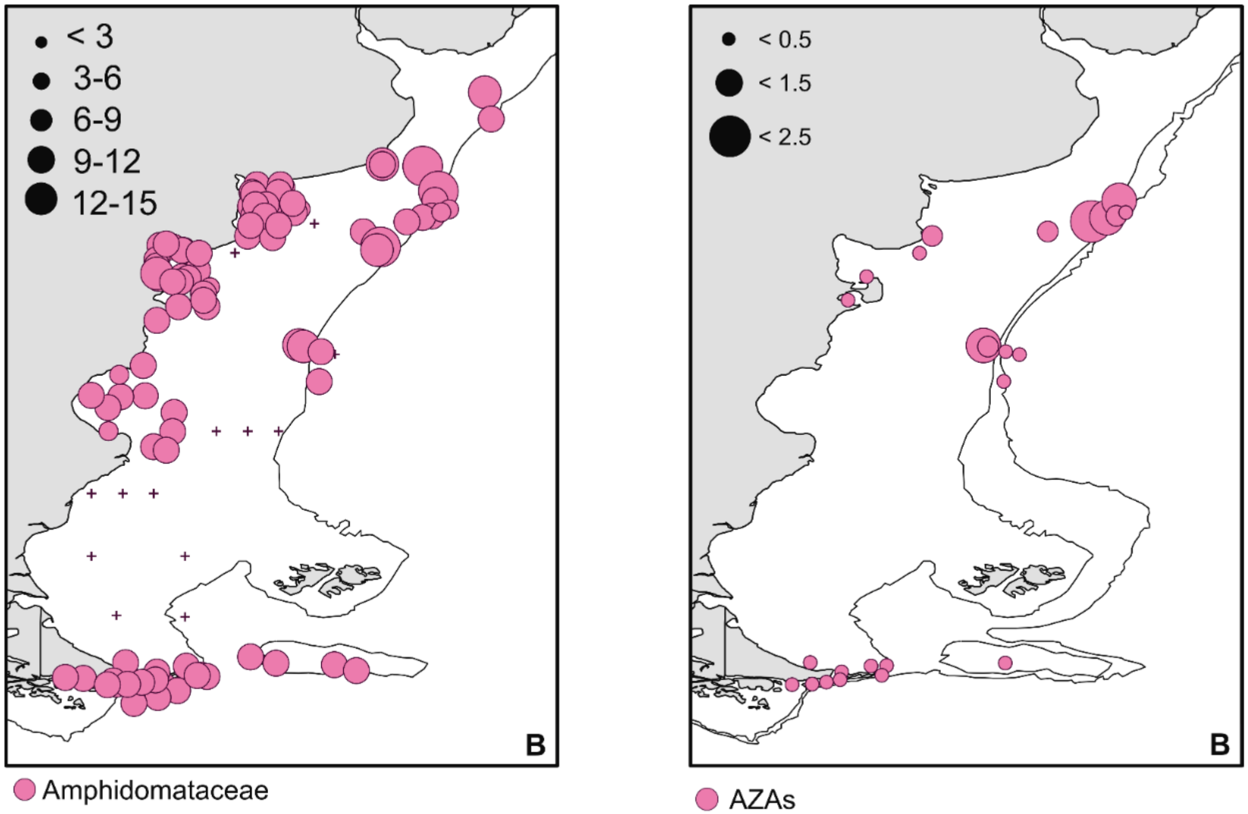

Bernd Krock, IADO, Bahía Blanca, 15th October 2024

# Occurrence of Toxins and their Producers

## Funded projects with AWI & IADO participation:

| | |
|---|---|
| 2012-2014 | BMBF-Mincyt "Interactions between biogeochemistry and plankton composition of the Argentine Continental Shelf" |
| 2013-2016 | EU: FP7-people-2012-IRSES, "IMCONet" |
| 2015-2016 | DAAD-MINCYT "Detection of phycotoxins in Monte Hermoso, Argentina" |
| 2015-2017 | BMBF-MINCYT "RETCEL" |
| 2014 | FONCyT PICT "Dinámica planctónica y vectorización de ictiotoxinas hacia consumidores de niveles superiores en tramas tróficas pelágicas de la región de El Rincón - Golfos Norpatagónicos" |
| 2017-2024 | BMBF "DynAMo" |
| 2020-2025 | EU: H2020-MSCA-RISE-2019 "CoastCarb" |
| 2021-2023 | DAAD-CONICET, "PhytoxNorpat" |

# Occurrence of Toxins and their Producers




Ramírez, F.J. et al. 2022 Harmful Algae 118: 102317.

# Occurrence of Toxins and their Producers

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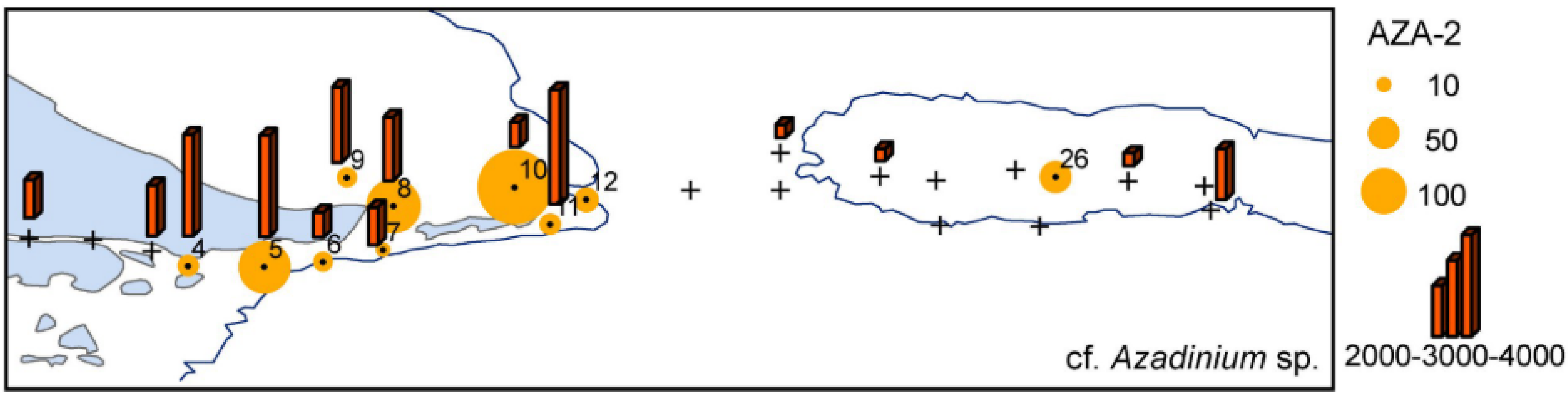


Guinder, V.A., et al. (2020). PLOS ONE 15(5): e0233156.

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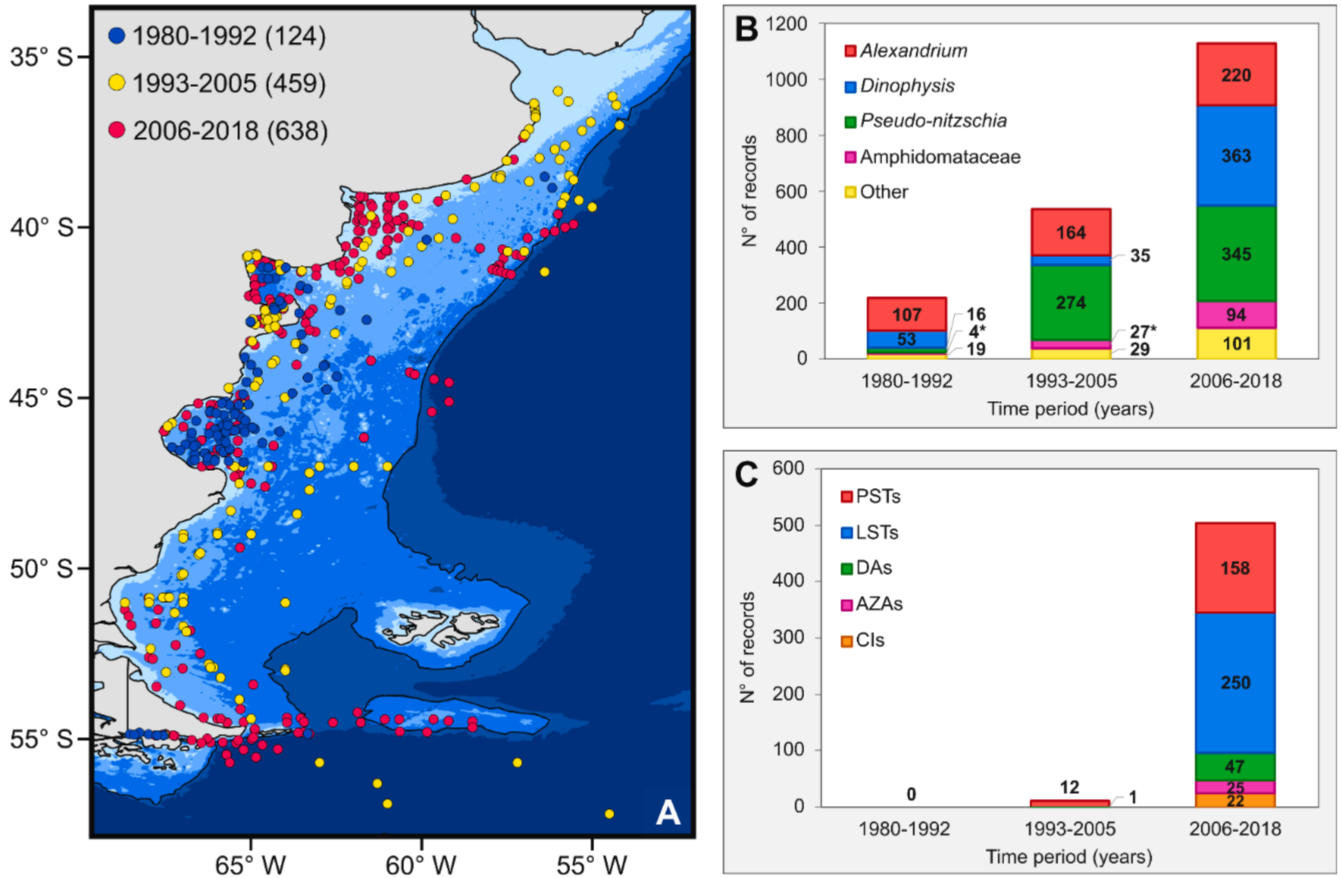
# Occurrence of Toxins and their Producers




Ramírez, F.J. et al. 2022 Harmful Algae 118: 102317.

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