Azaspiracids, an Expanding Group of Shellfish Poisoning Toxins

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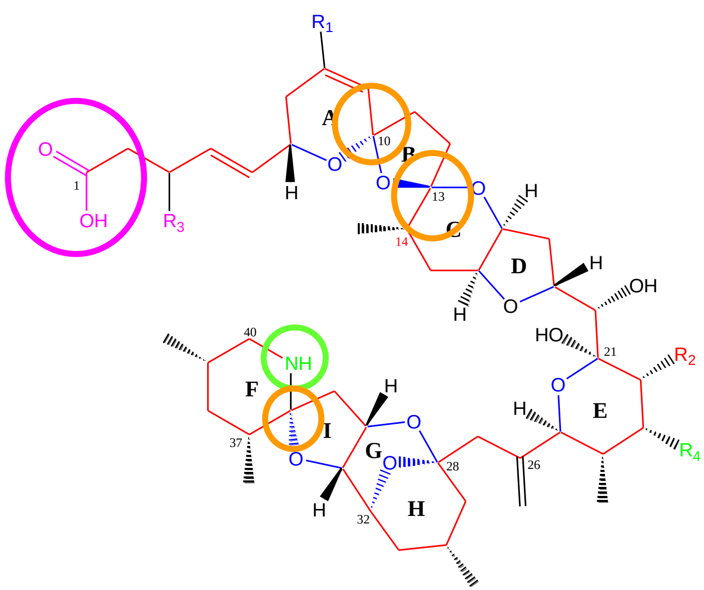
1. Search for toxigenic species

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)



azaspiracid

Polyketide:
linear carbon skeleton
with cyclic ether bridges
amino function
chemical nomenclature:
aza = secondary amine
spiro function
acid

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1. Search for toxigenic species

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

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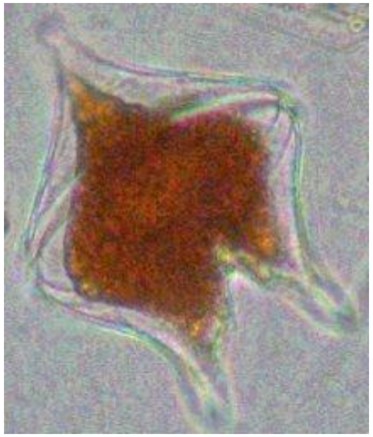
Abstract

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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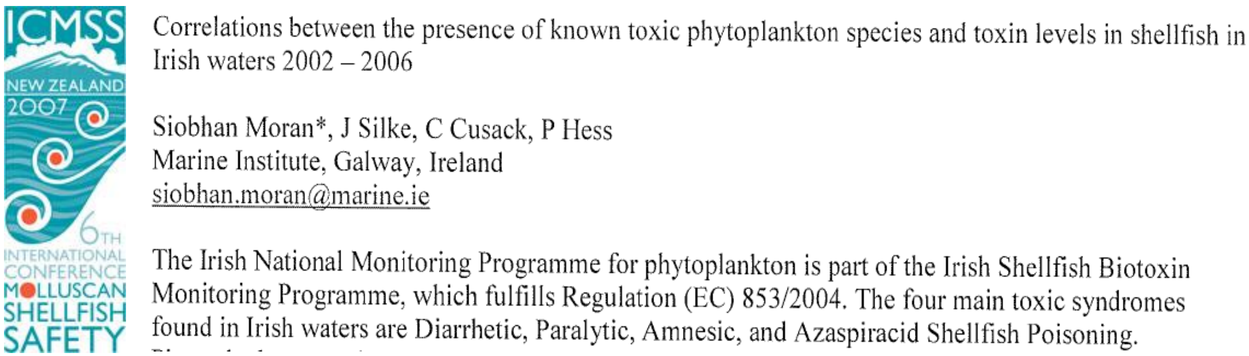

ICMSS NEW ZEALAND 2007 6TH INTERNATIONAL CONFERENCE MOLLUSCAN SHELLFISH SAFETY

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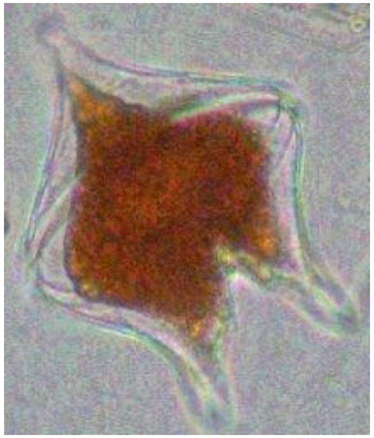
The Irish National Monitoring Programme for phytoplankton is part of the Irish Shellfish Biotoxin Monitoring Programme, which fulfills Regulation (EC) 853/2004. The four main toxic syndromes found in Irish waters are Diarrhetic, Paralytic, Amnesic, and Azaspiracid Shellfish Poisoning.



Protoperidinium crassipes

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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Scientific expedtion on the North Sea June/July 2007

+

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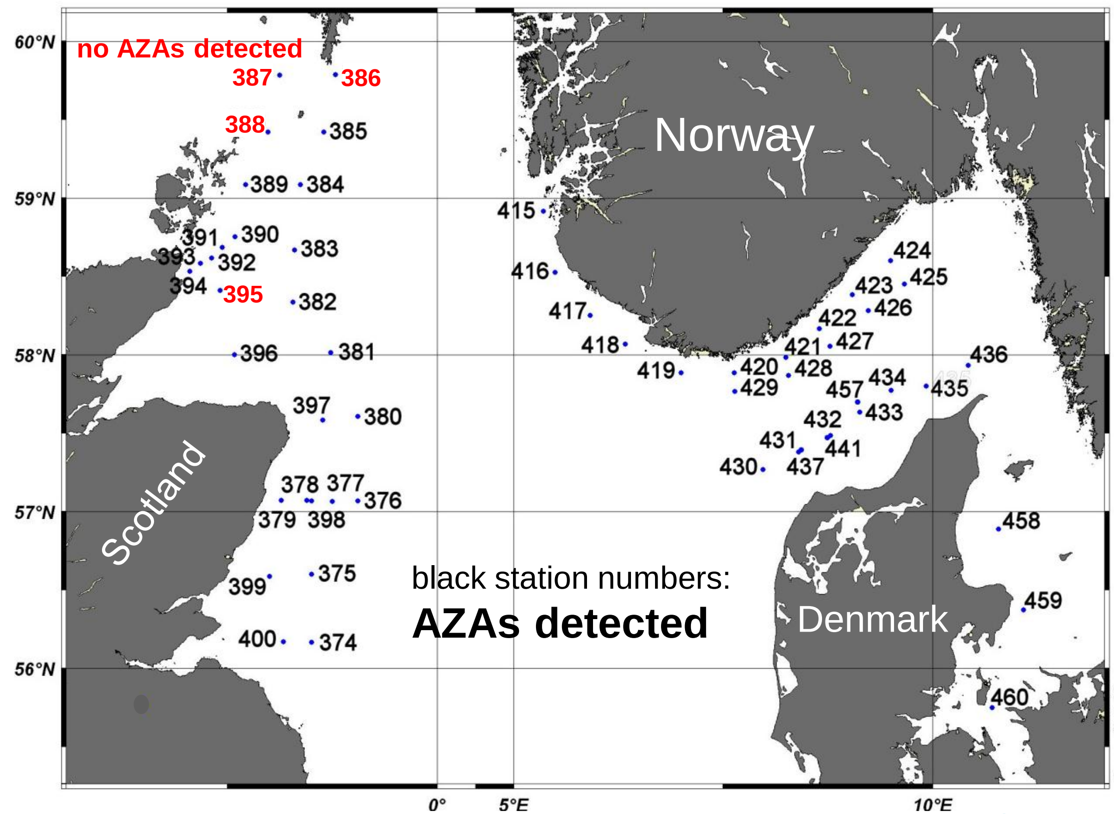

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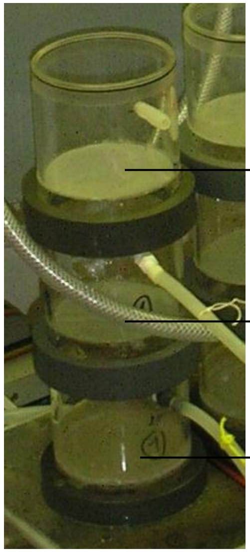
1. Search for toxigenic species

Plankton fractionation

Plankton net, pore size 20 µm



Filter array

200 µm (zooplankton)

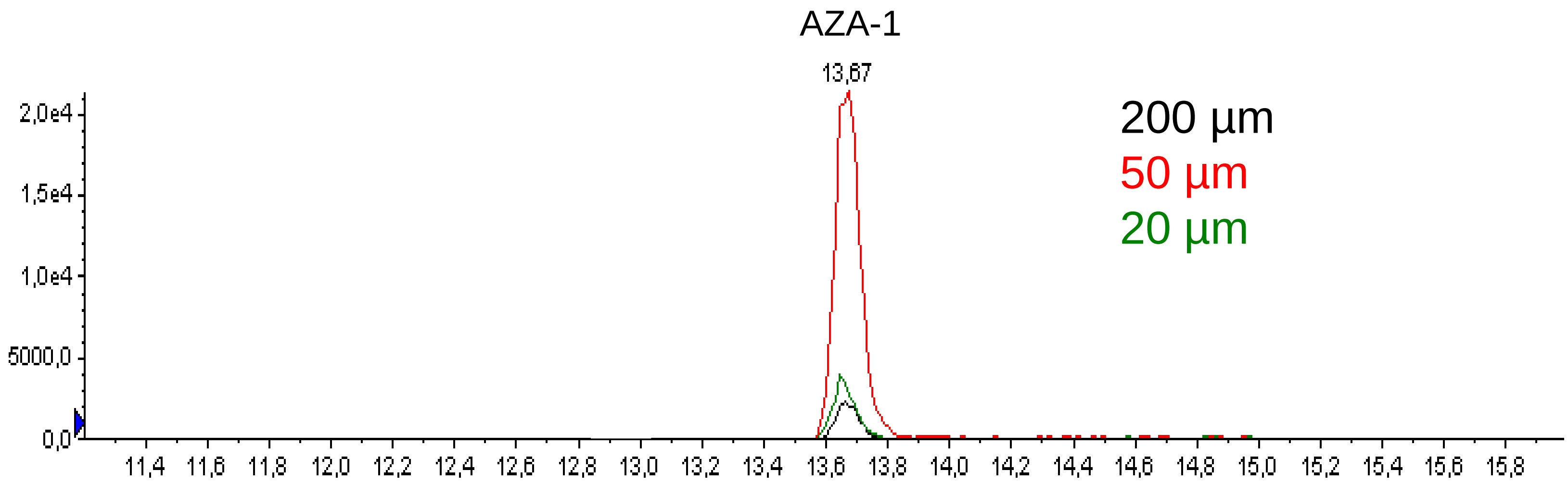
50 µm (big phytoplankton)

20 µm (small phytoplankton)

LC-MS

2. Search for the AZA producer

Size fraction test



Look into 50 µm plankton fraction

Favella ehrenbergii

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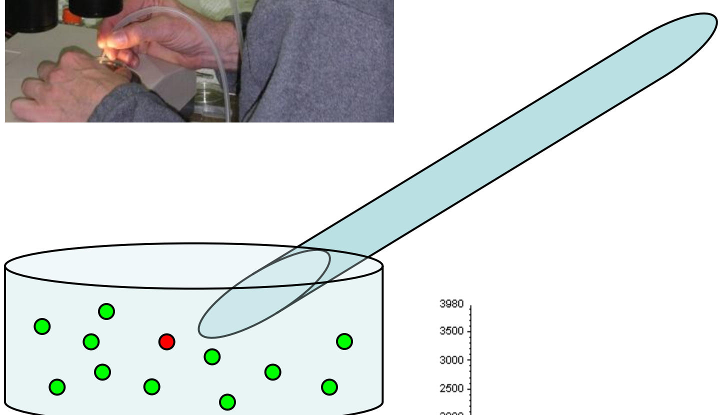
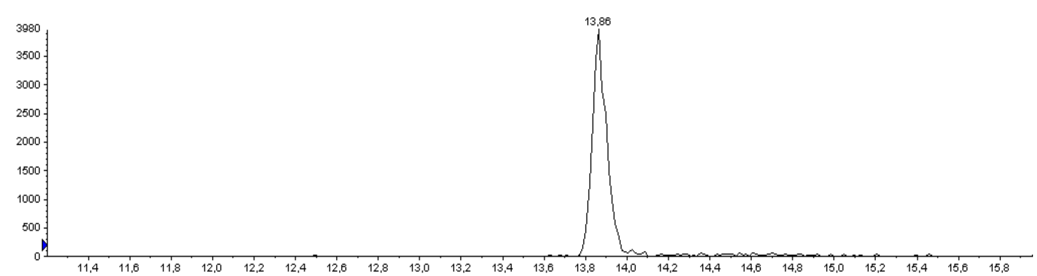
1. Search for toxigenic species

Putative target organism isolation

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

Liquid chromatography-mass spectrometry (LC/MS)



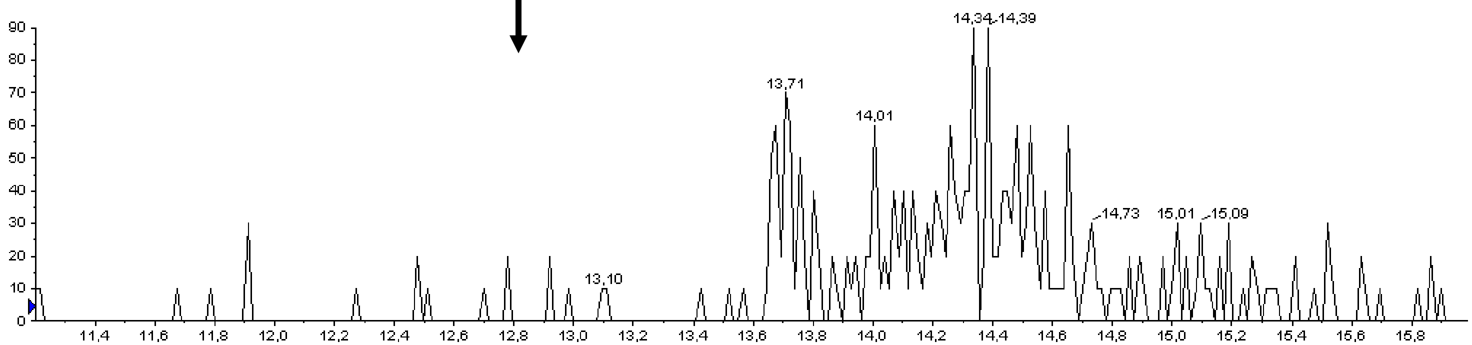


1. Search for toxigenic species

Feeding experiment

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)



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

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2. Search for the AZA producer

New hypothesis: producing organisms are < 20 µm

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

=> screening of size fractions < 20 µm for AZA

1. Search for toxigenic species

Screening of plankton < 20 µm for AZA

Rosette sampler
(unfiltered water samples)

Filtration over 20 µm gauze

Removal of plankton > 20 µm

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

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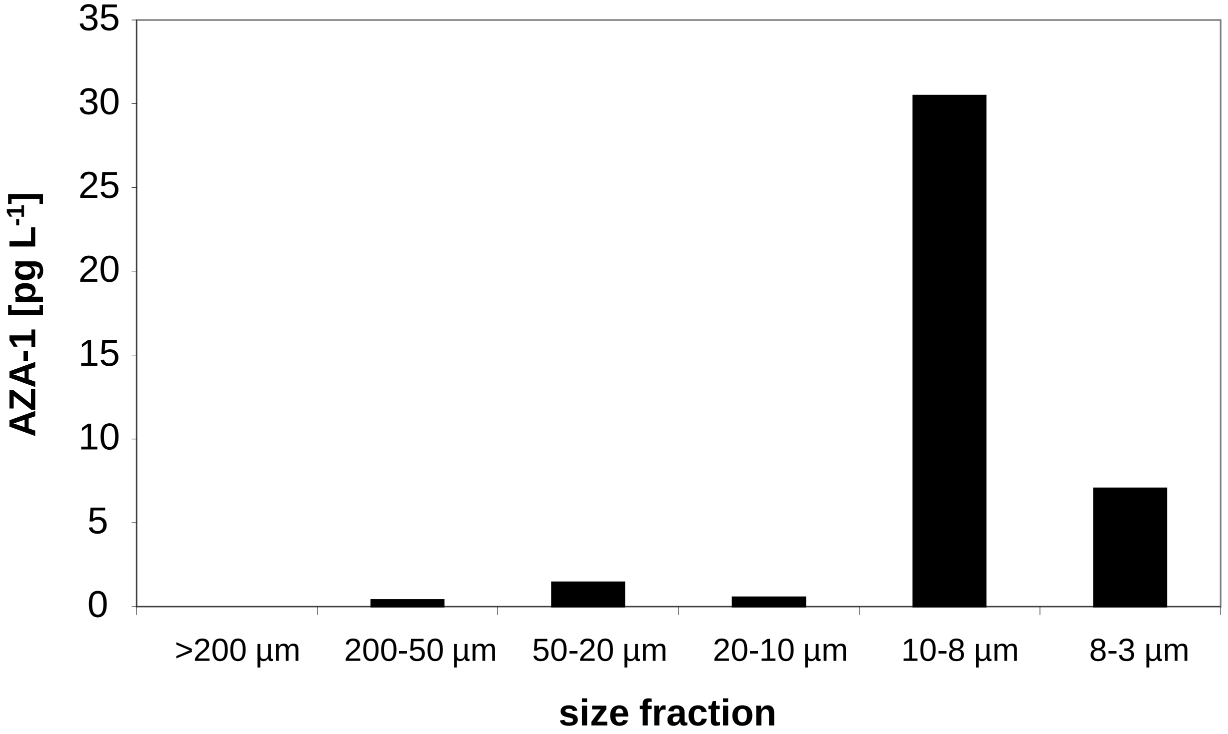

=> the AZA producer is approximately 10 µm big

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

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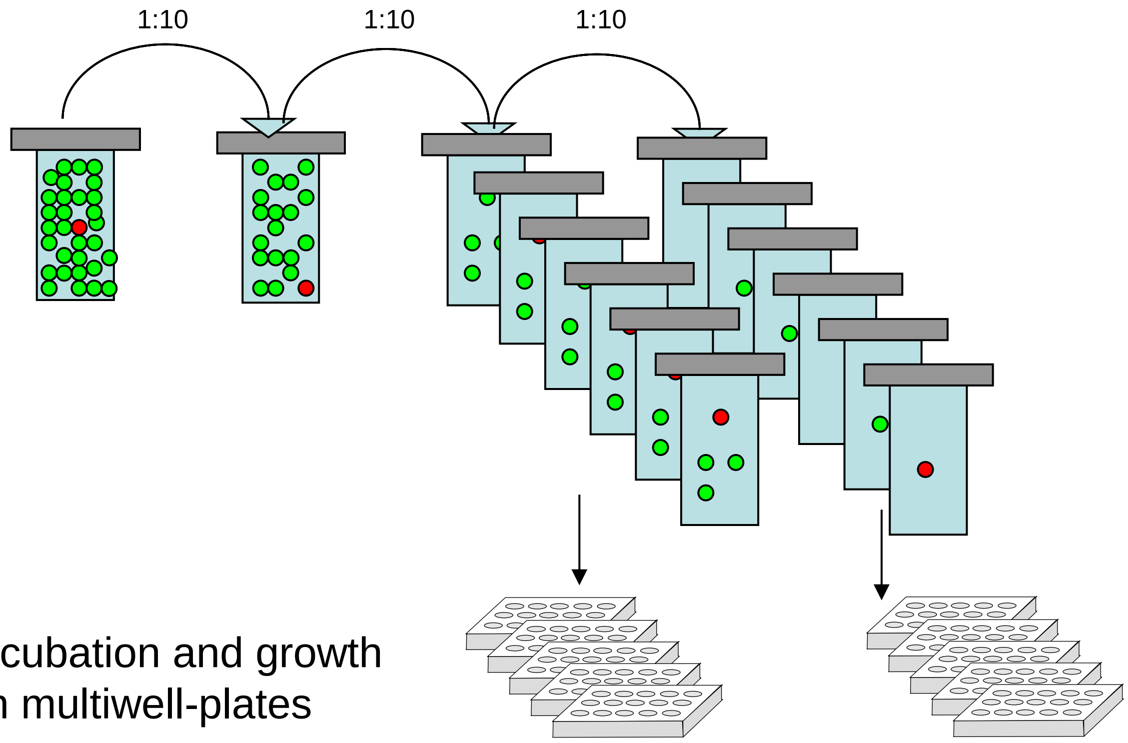
Species isolation

Serial dilution method



Incubation and growth
in multiwell-plates

1. Search for toxigenic species

Isolate screening

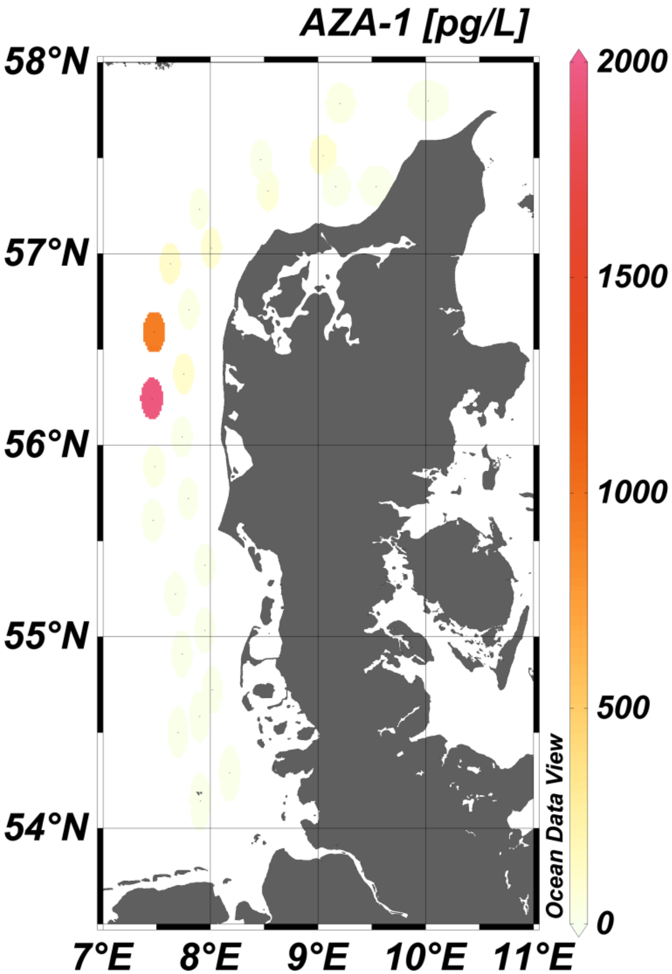
Out of 240 isolates tested

only one culture “3 D9“ contained AZA

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

1. Search for toxigenic species

Expedition along the Danish west coast July 2008



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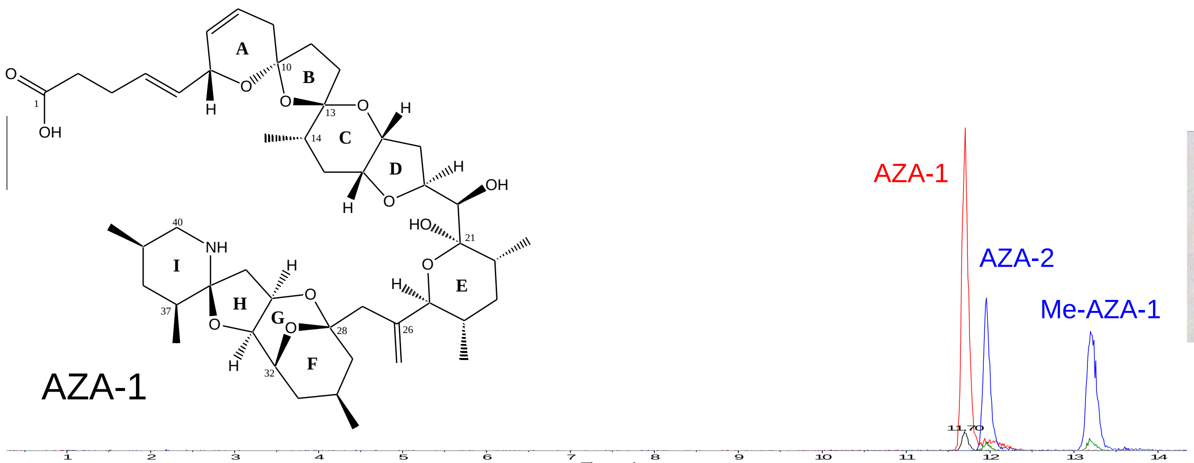
1. Search for toxigenic species

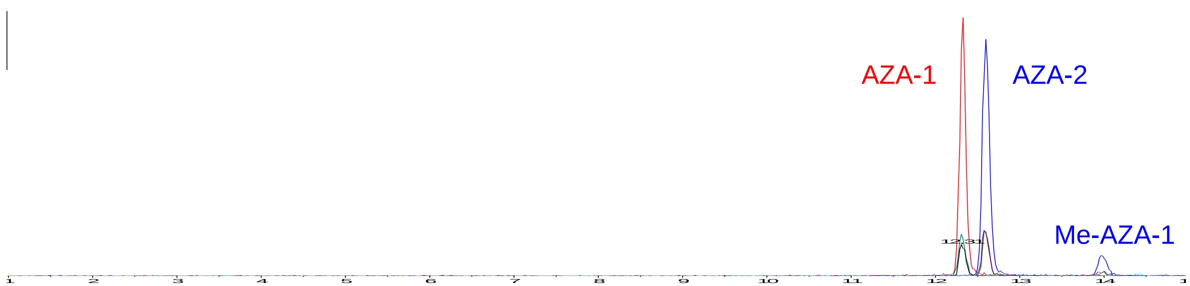
Toxin profile of A. spinosum



Scottish strain 3D9 (2007)

***m/z* 842>824**

***m/z* 856>838**



Danish strain UTH E2 (2008)

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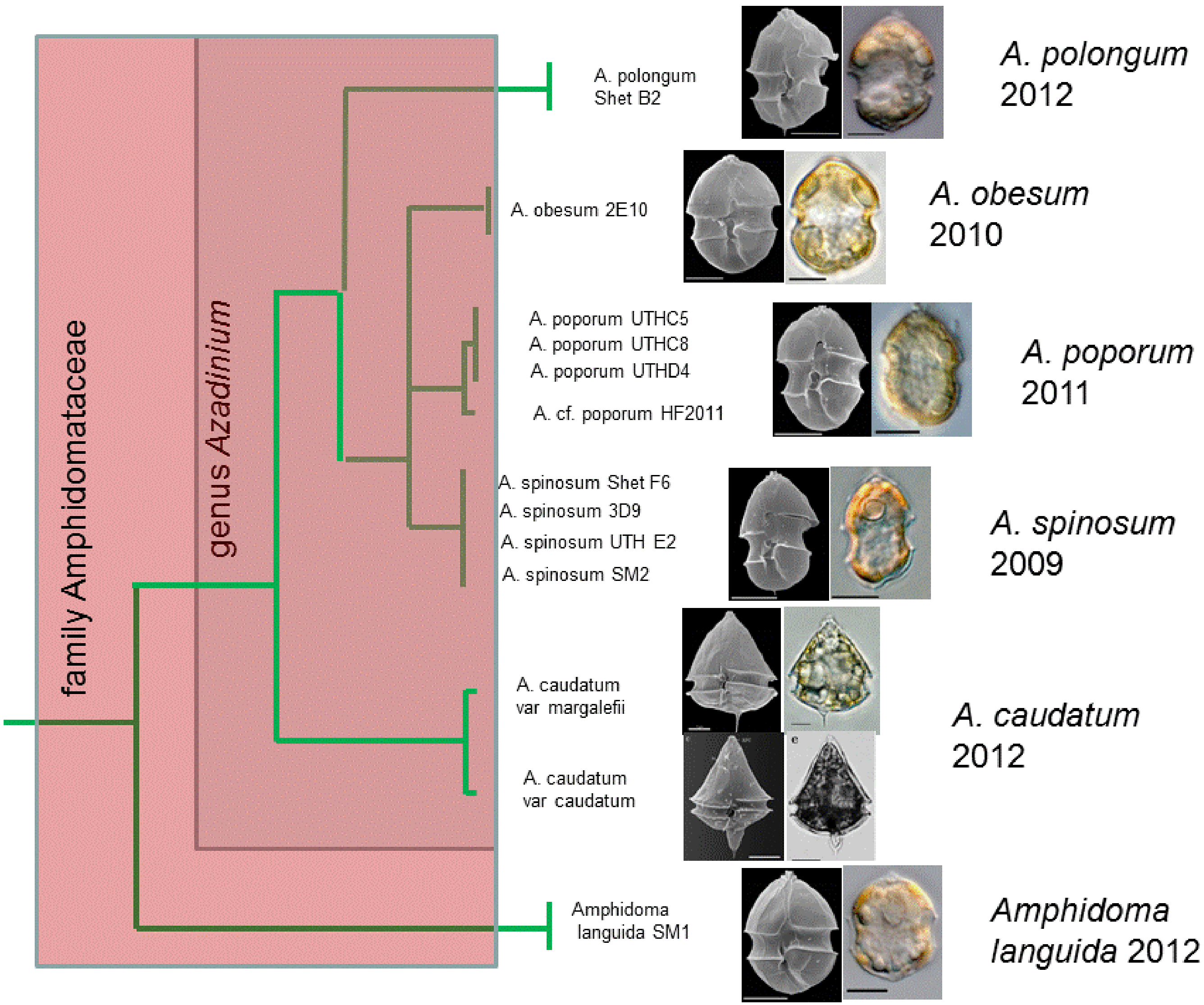

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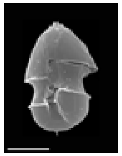 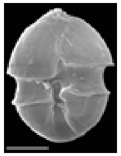 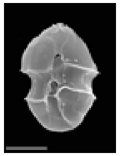 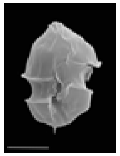 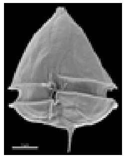 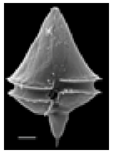 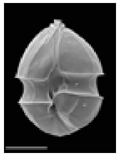

	Azadinium spinosum	*Azadinium obesum*	*Azadinium poporum*	*Azadinium polongum*	*A. caudatum* var. *margalefii*	*A. caudatum* var. *caudatum*	*Amphidoma languida*
size	13.8 x 8.8	15.3 x 11.7	13.0 x 9.8	13.0 x 9.7	31.1 x 22.4	41.7 x 28.7	13.9. x 11.9
length/width ratio	1.6	1.3	1.3	1.3	1.2	1.2	1.2
Pyrenoid	1; epicone	-	Several (4?), epi- and hypocone	-	-	-	1; epicone
Spine	+	-	-	+	**Short horn, long spine**	**Long horn, short spine**	
Ventral pore	+	+	-	+	-	+	+
Pore on po-plate	-	-	+	-	+	-	-
Shape pore-plate	**round-elipsoid**	**round-elipsoid**	**round-elipsoid**	**elongated**	**round-elipsoid**	**round-elipsoid**	**round-elipsoid**
Plate 1´´ in contact to plate 1a	+	-	+	+	+	+	+
Plate 6´´ in contact to plate 3a	-	-	-	-	+	+	-
Azaspiracids	+	-	+	-	-	-	+

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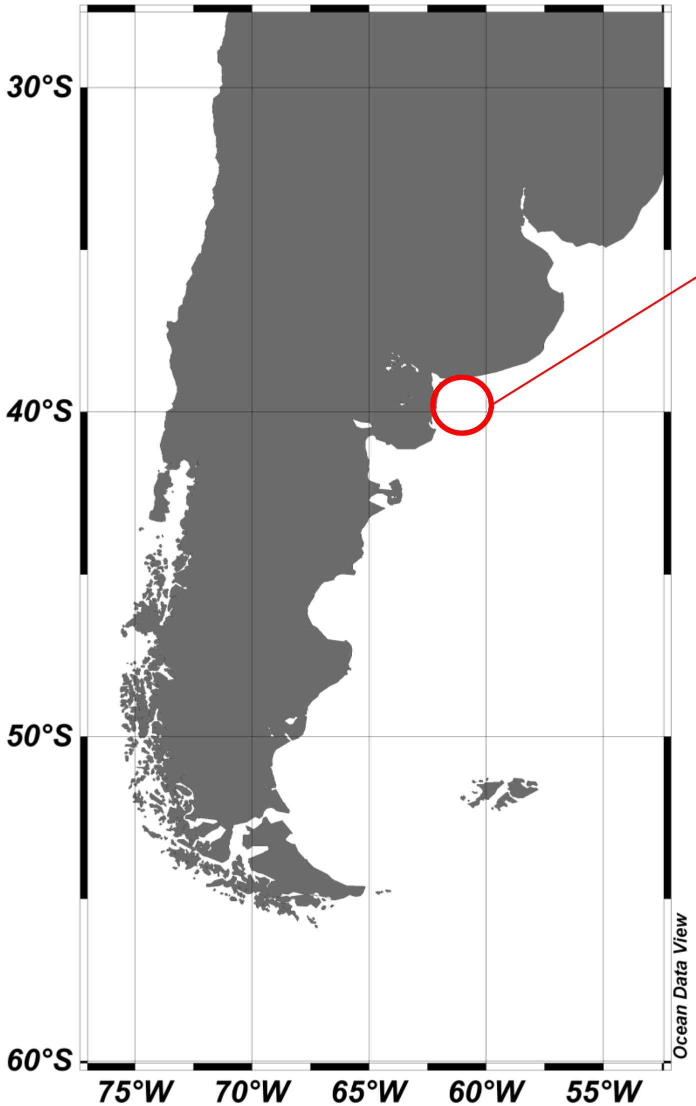

Recently 10 *Azadinium* cultures established by hatching resting cysts from sediment samples

All of them produce AZA-2
And other not yet identified AZAs

Species? work in progress

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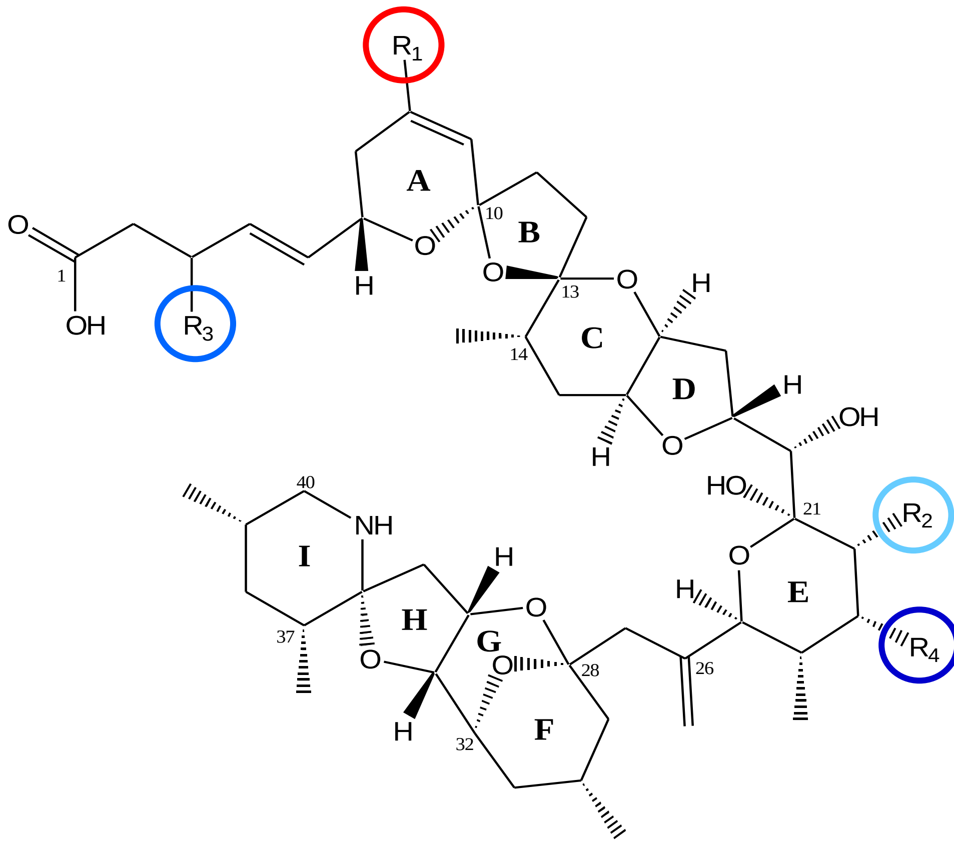
2. Novel toxins



Toxin	R_1	R_2	R_3	R_4	$[M+H]^+$
AZA-1	H	CH_3	H	H	842
AZA-2	CH_3	CH_3	H	H	856
AZA-3	H	H	H	H	828
AZA-4	H	H	OH	H	844
AZA-5	H	H	H	OH	844
AZA-6	CH_3	H	H	H	842
AZA-7	H	CH_3	OH	H	858
AZA-8	H	CH_3	H	OH	858
AZA-9	CH_3	H	OH	H	858
AZA-10	CH_3	H	H	OH	858
AZA-11	CH_3	CH_3	OH	H	872

To date more than 20 structural azaspiracid variants are known

Rehmann et al. (2008) Rapid Commun. Mass Spectrom., 22, 549-558

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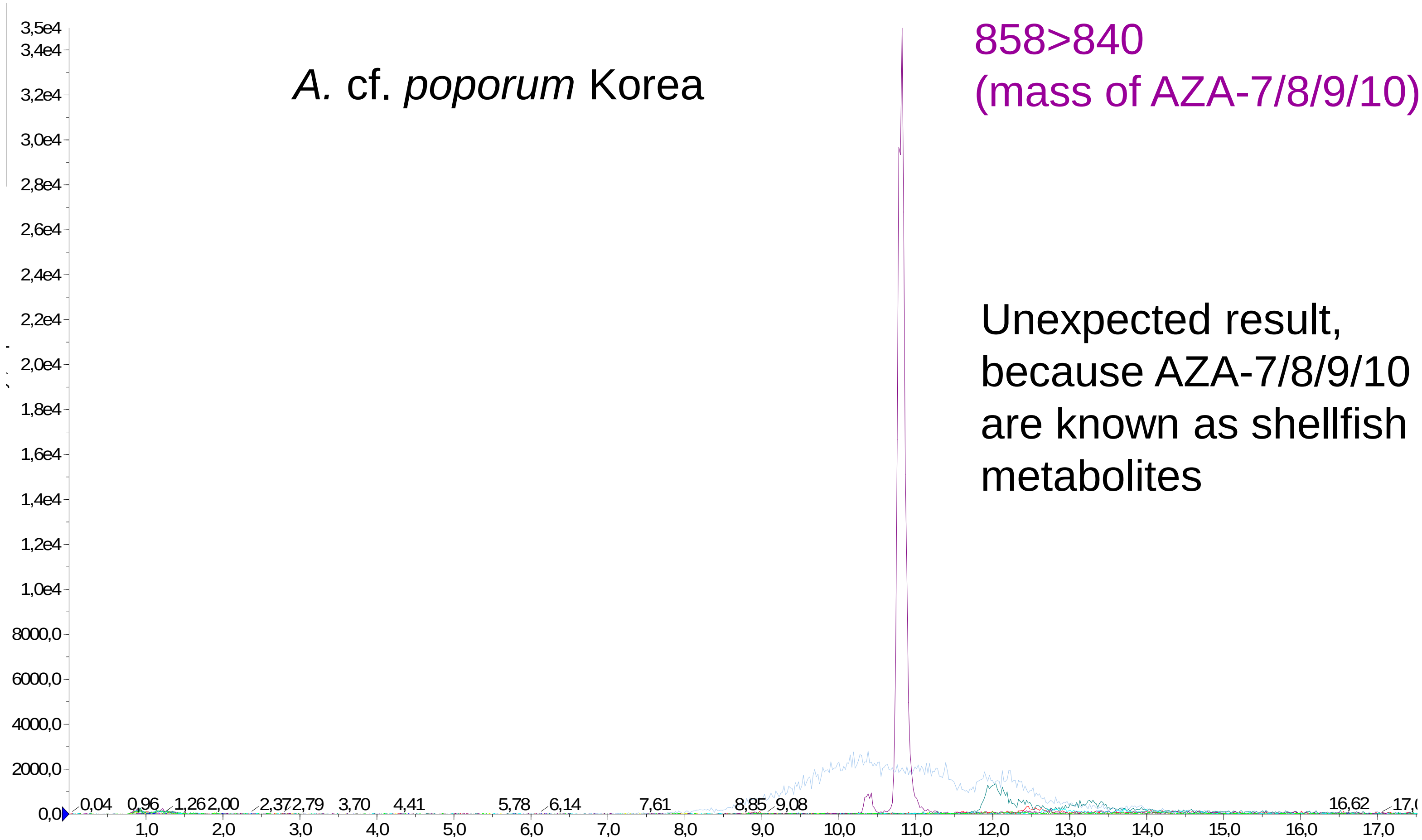
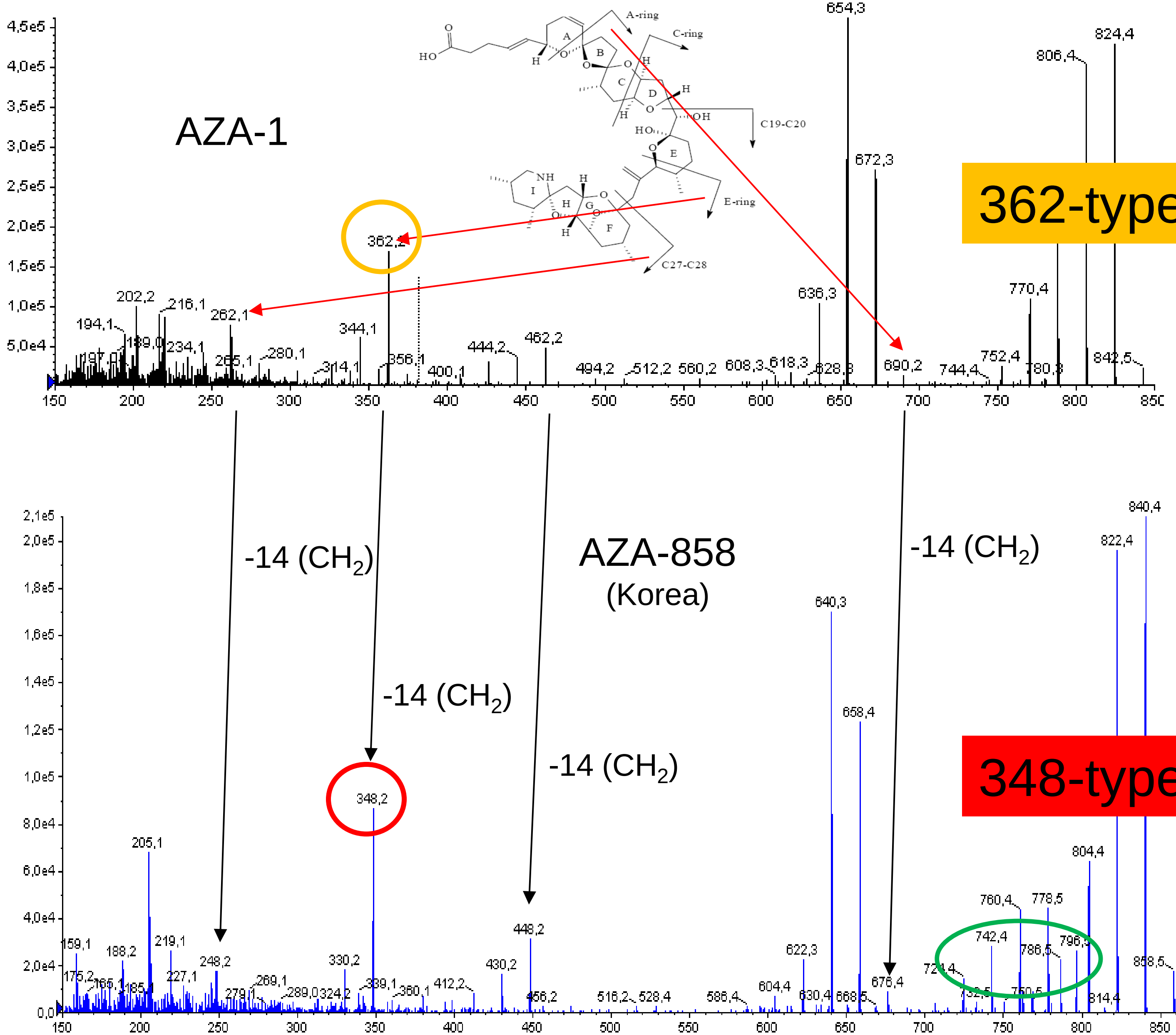
Test for AZAs (SRM):



858>840
(mass of AZA-7/8/9/10)

Unexpected result, because AZA-7/8/9/10 are known as shellfish metabolites

2. Novel toxins



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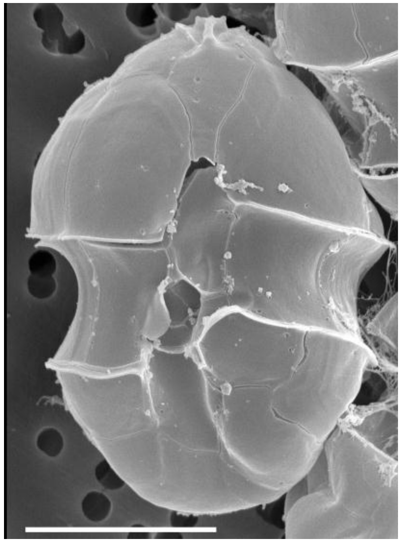
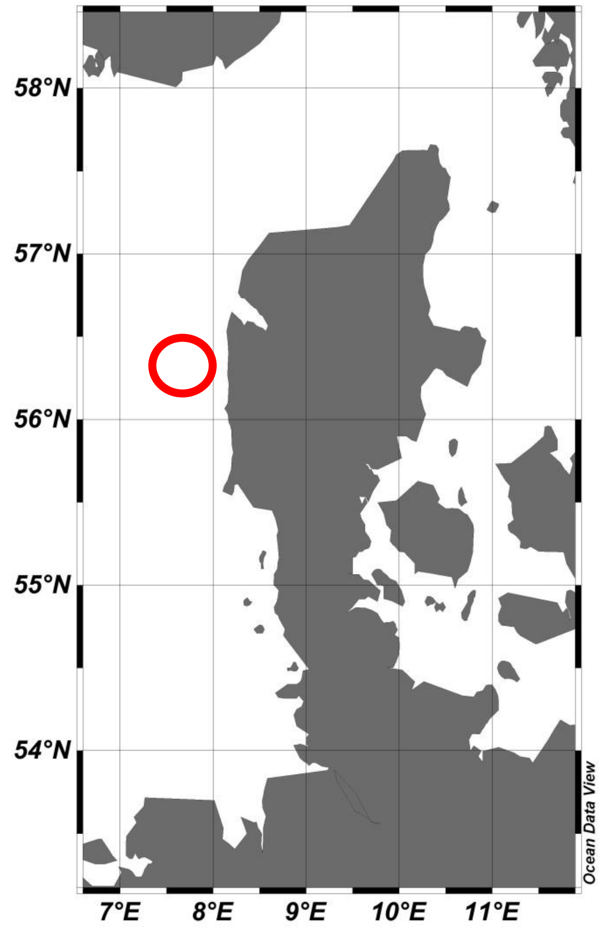


A. poporum C5 North Sea



Danish Coast at
56° 14.52’ N, 07° 27.54’ E

Negative for known AZAs

Tillmann et al. 2011. *Eur. J. Phycol.*, **46**, 74 - 87.

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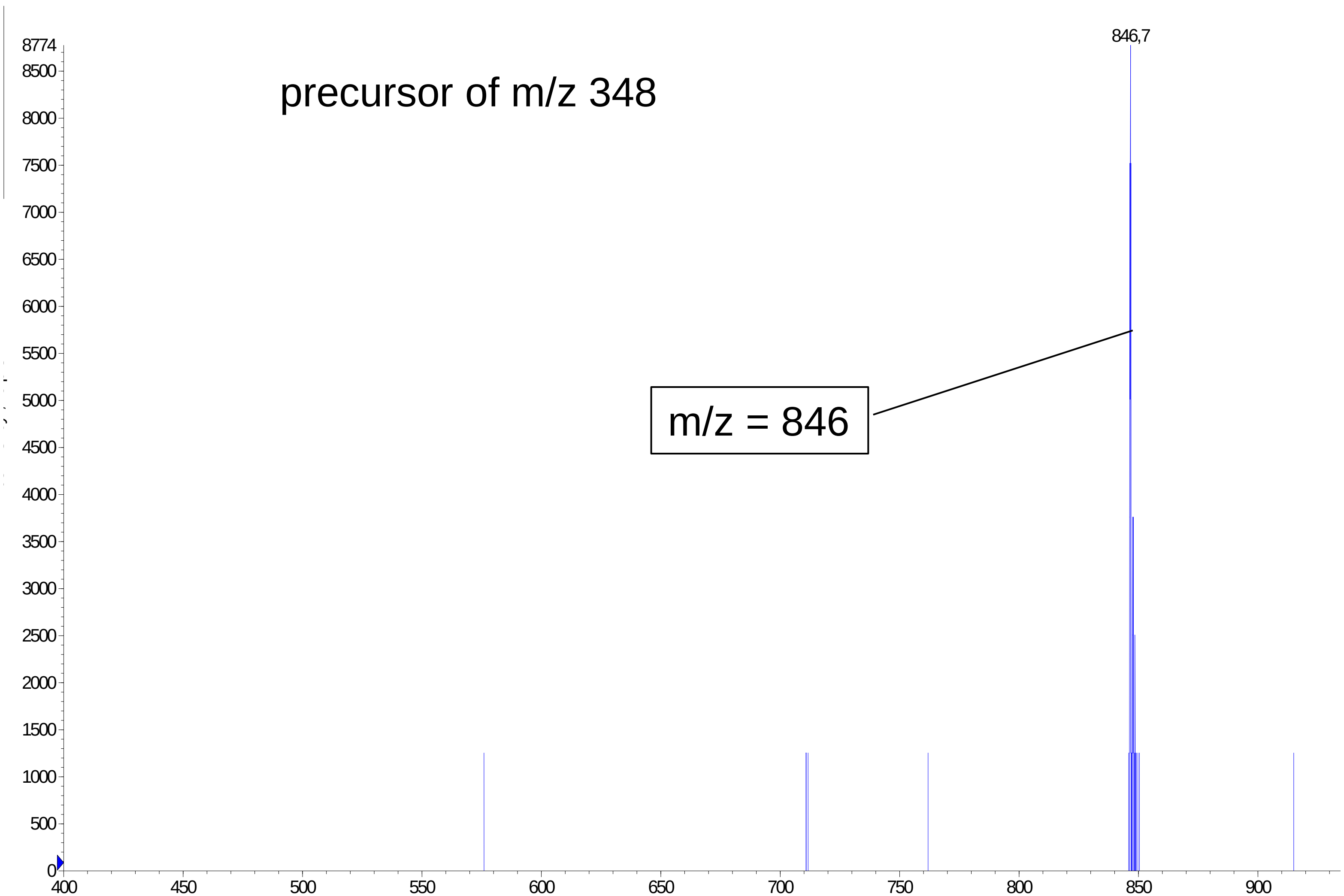

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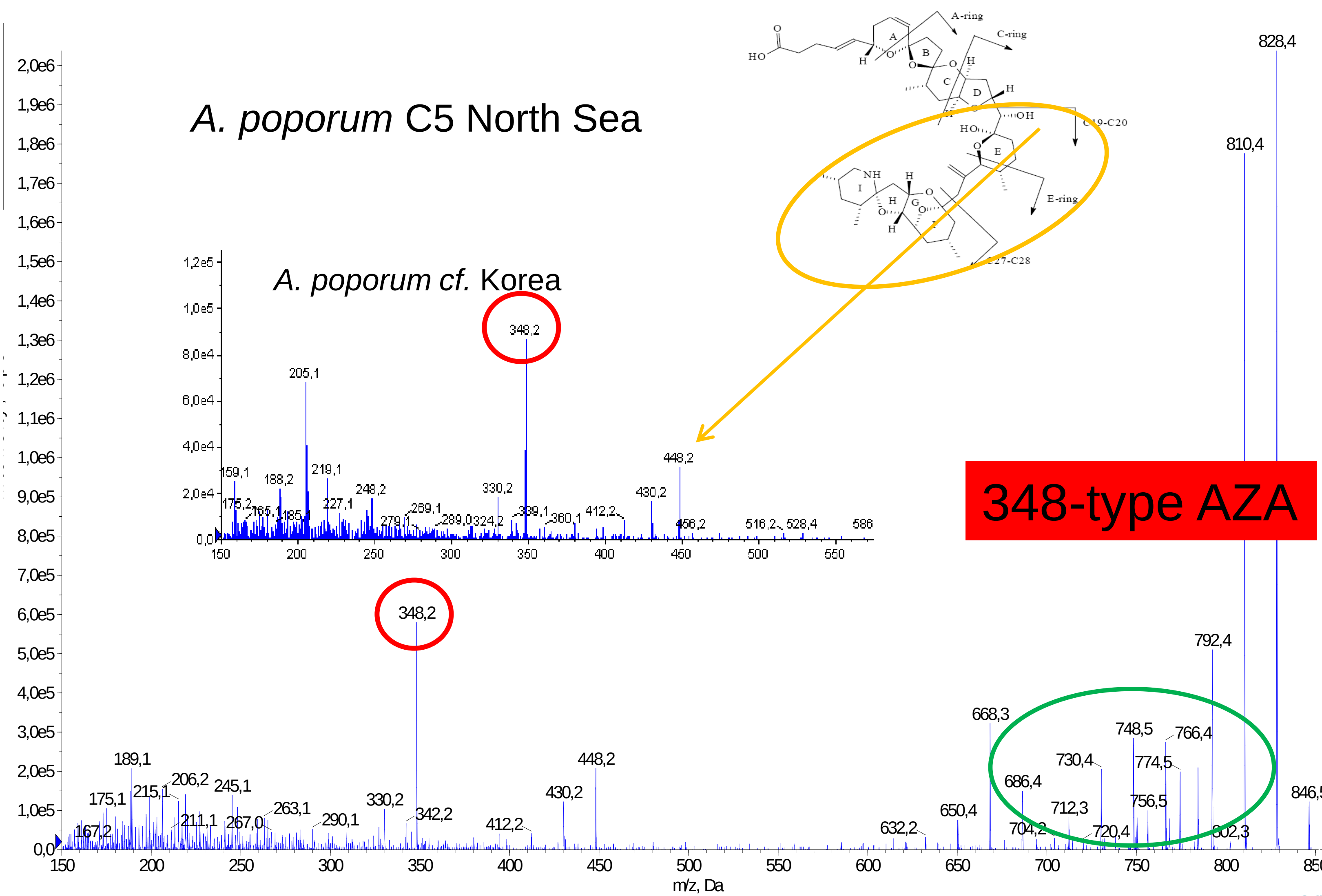
2. Novel toxins



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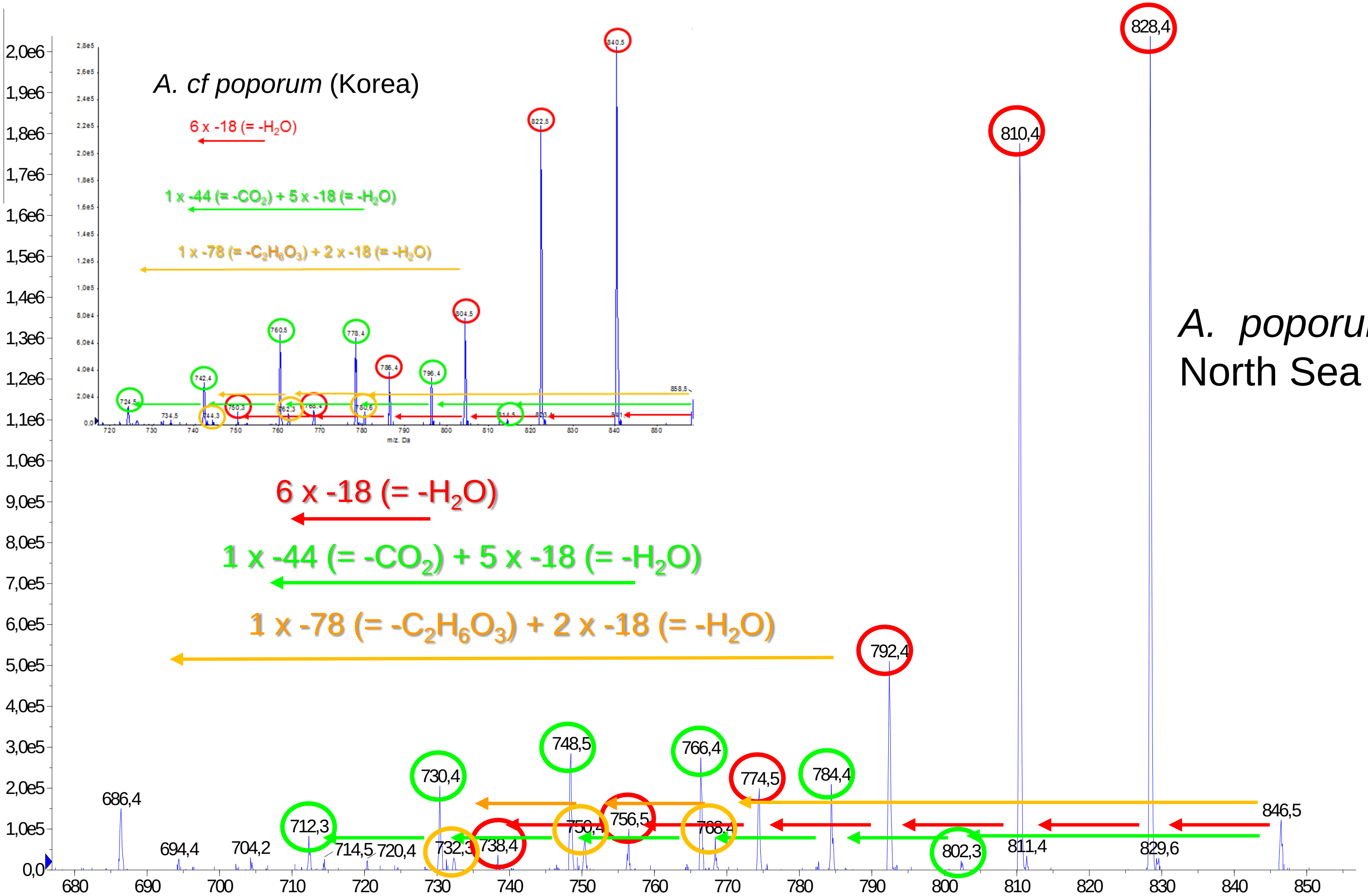
2. Novel toxins



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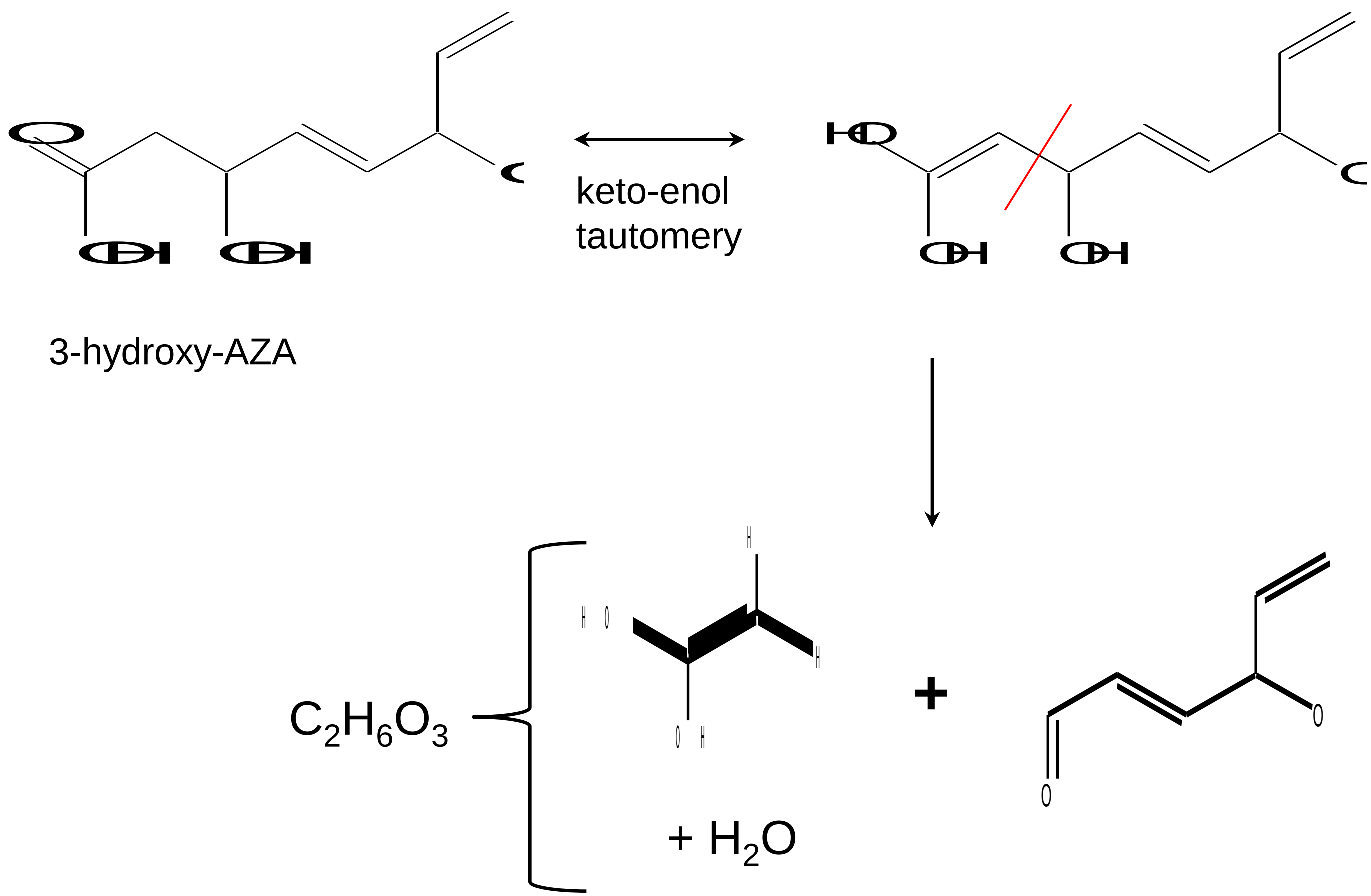
2. Novel toxins

Fragmentation pattern for the cleavage of m/z 78 (= $C_2H_6O_3$)



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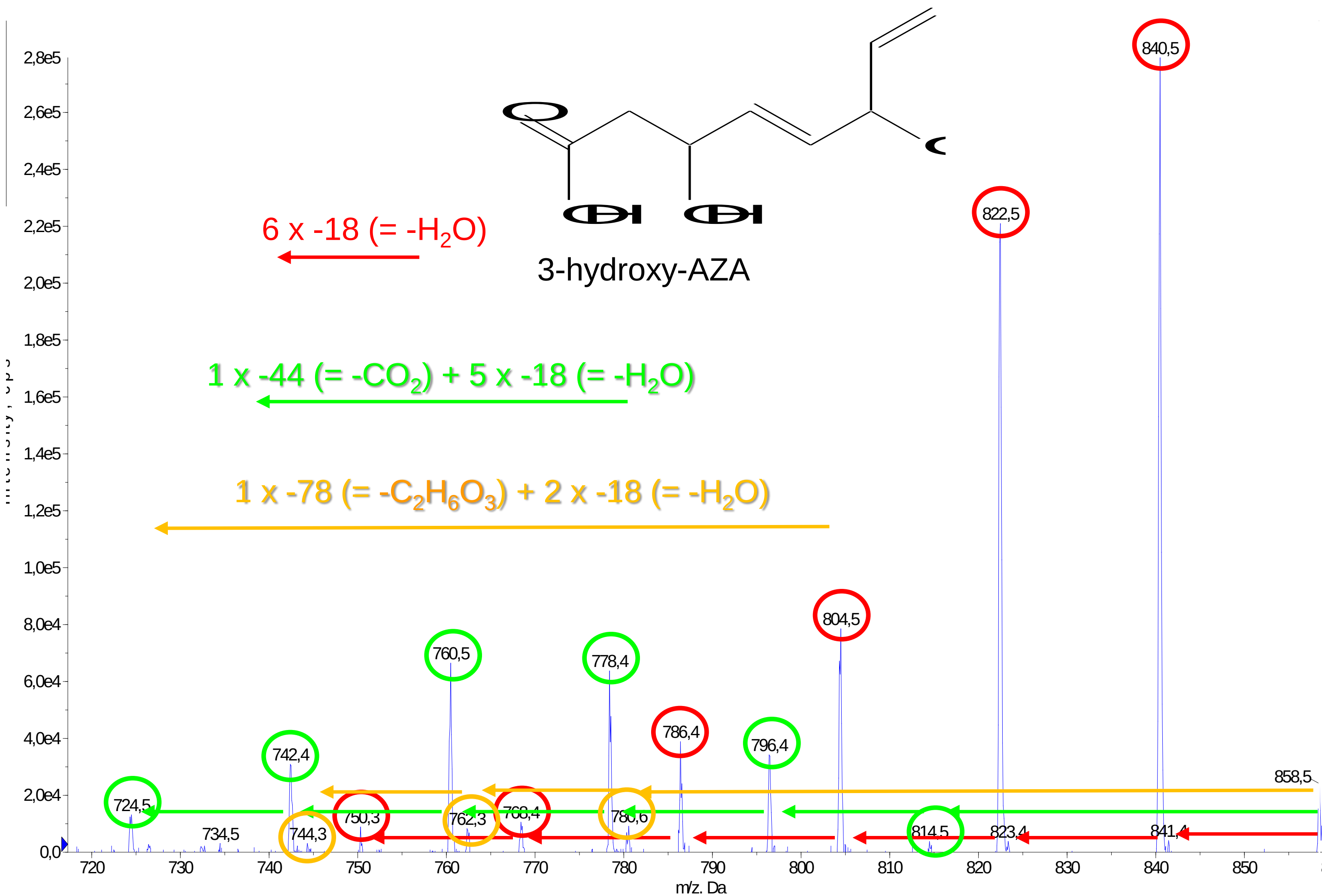
3. Novel toxins



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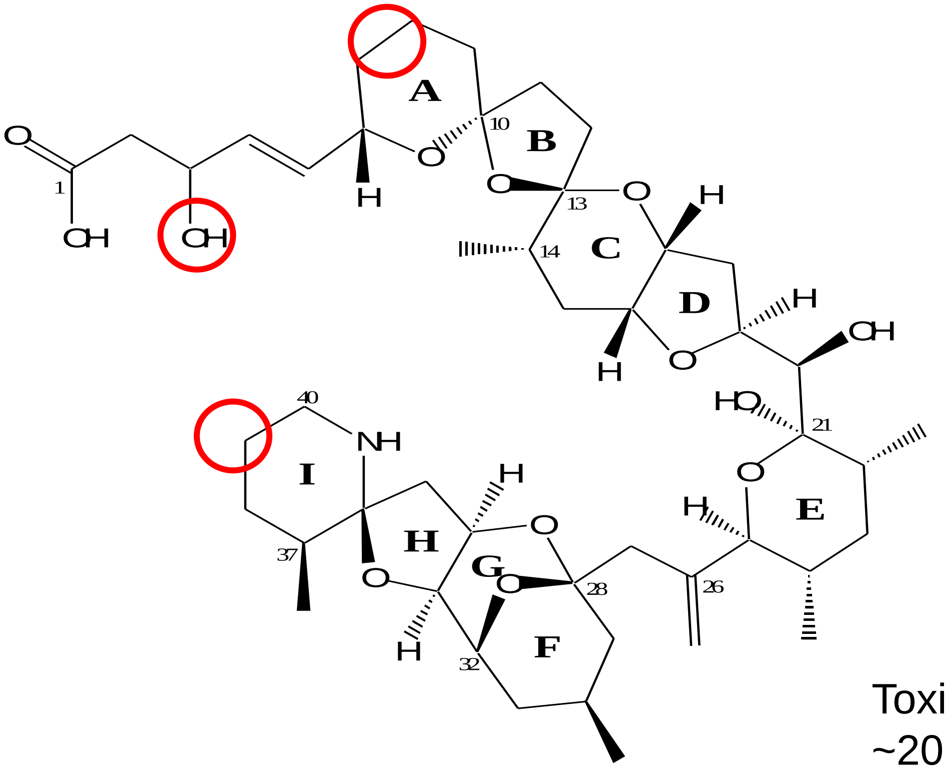
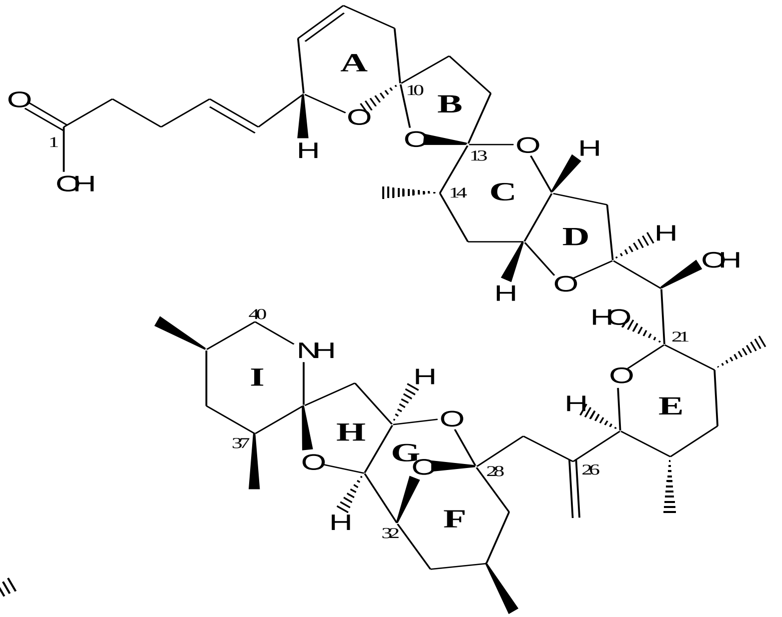
2. Novel toxins

A. poporum C5 North Sea





AZA-1

Toxin cell quota:
~20 fg/cell

AZA-846: 39-desmethyl-7,8-dihydro-3-hydroxy-AZA-1
(Krock et al. in preparation)

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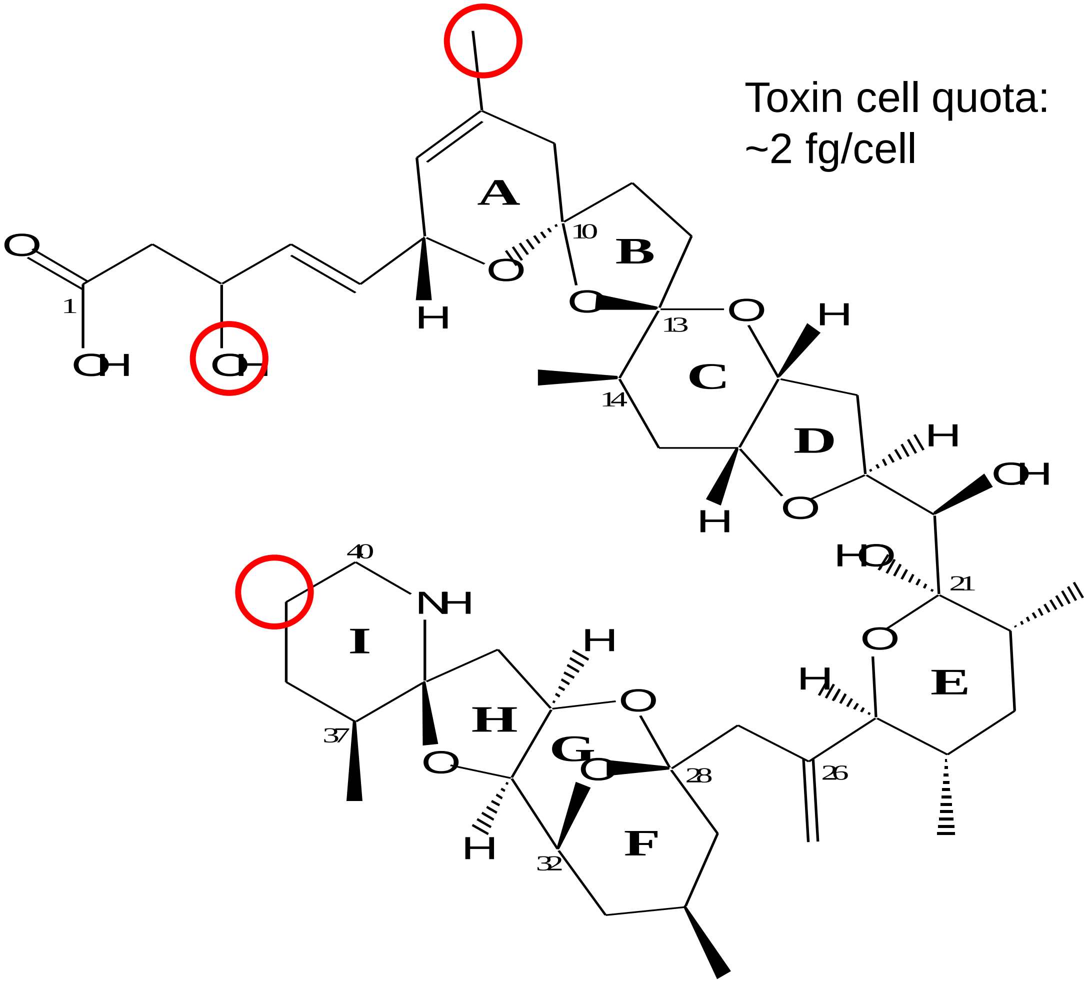
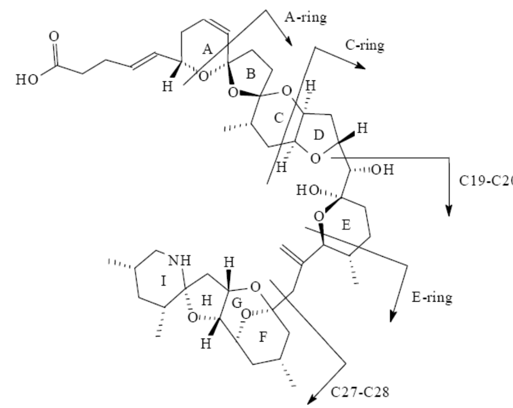


Toxin cell quota:
~2 fg/cell



AZA-1

sum formulas
as determined by HRMS:

AZA-1: $C_{47}H_{71}NO_{12}$
AZA-858: $C_{47}H_{71}NO_{13}$

AZA-858 = 39-desmethyl-3-hydroxy-AZA-2
(Krock et al. in preparation)

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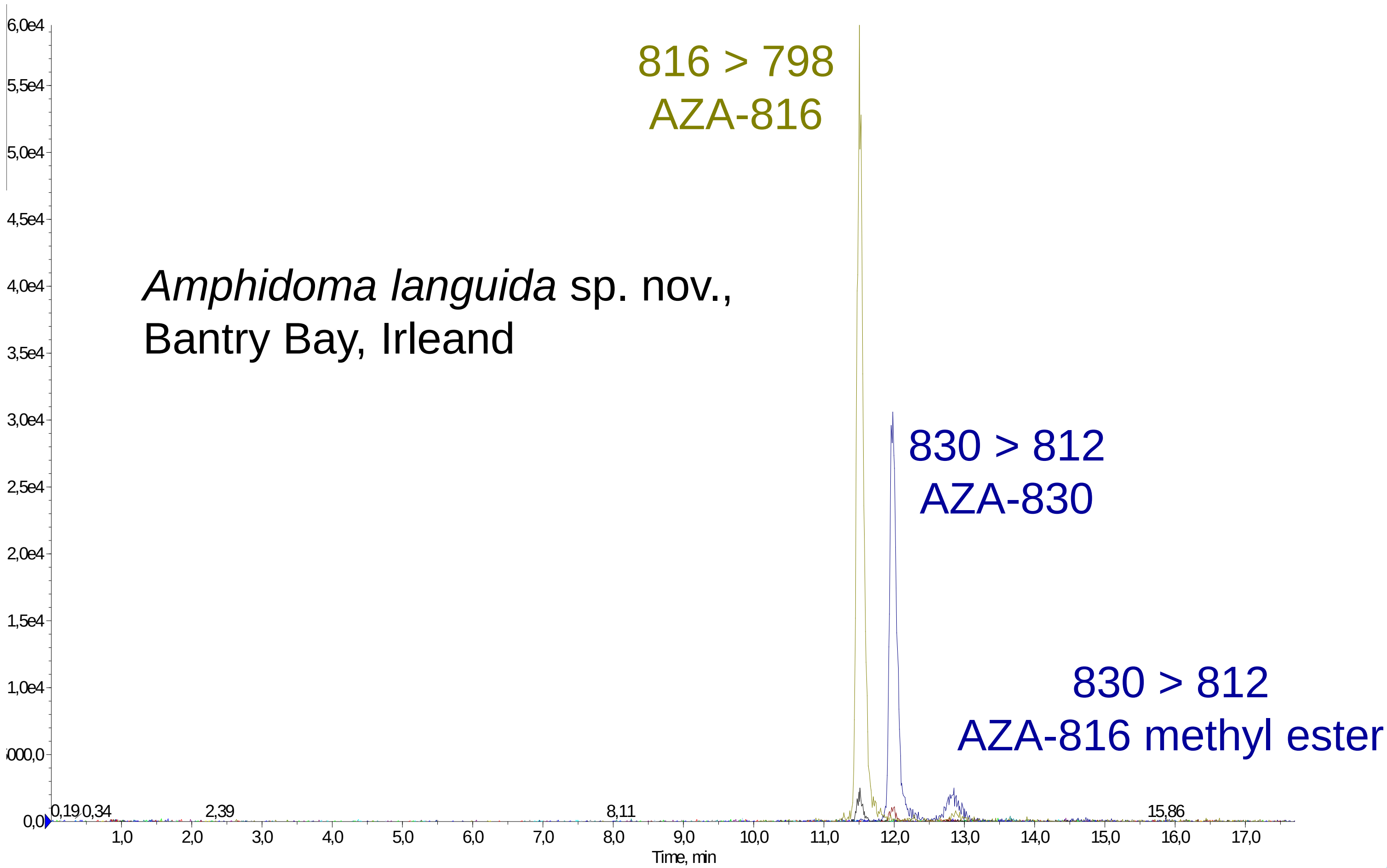
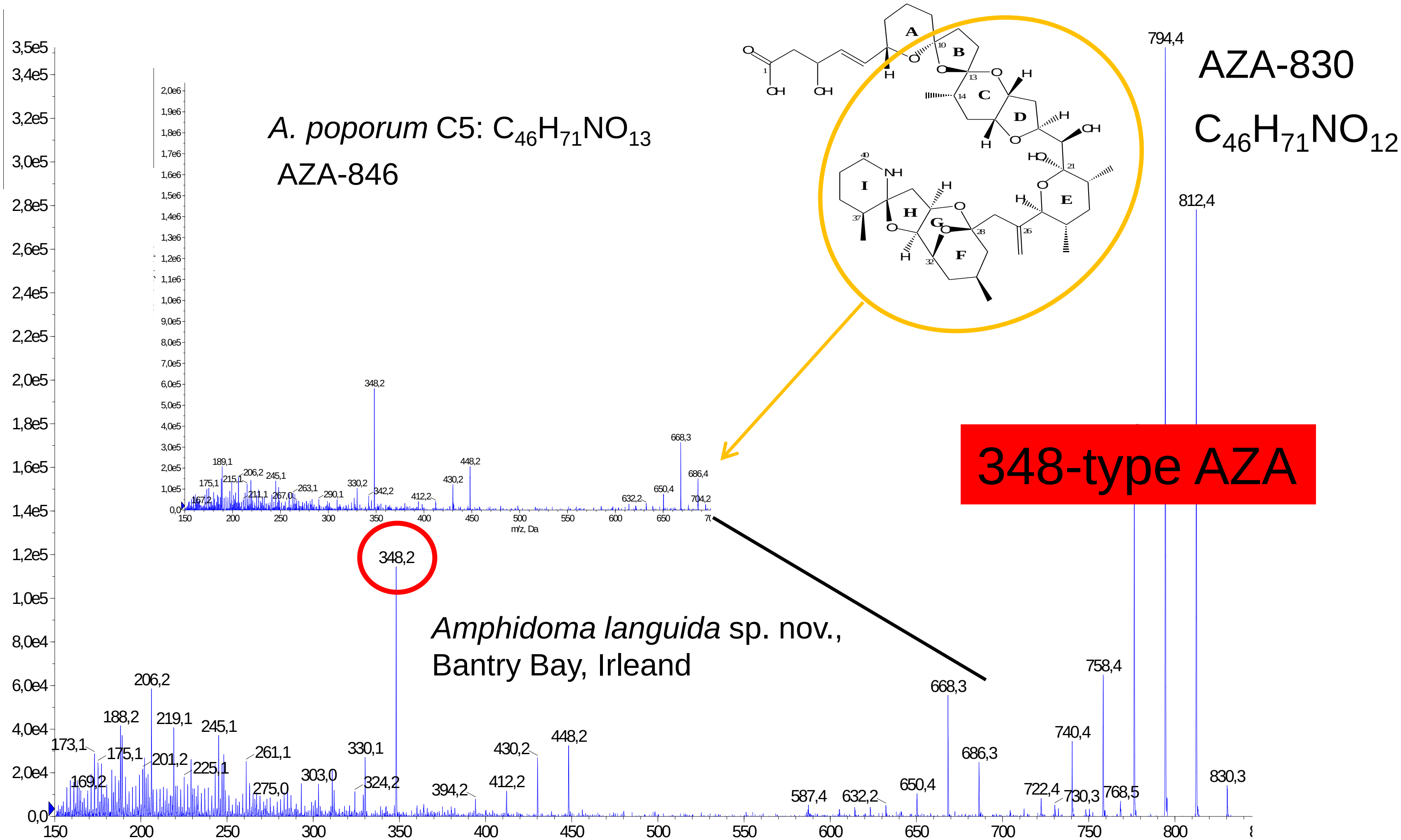


Tillmann U. et al. 2012 *Protist* 163, 701-719.

2. Novel toxins



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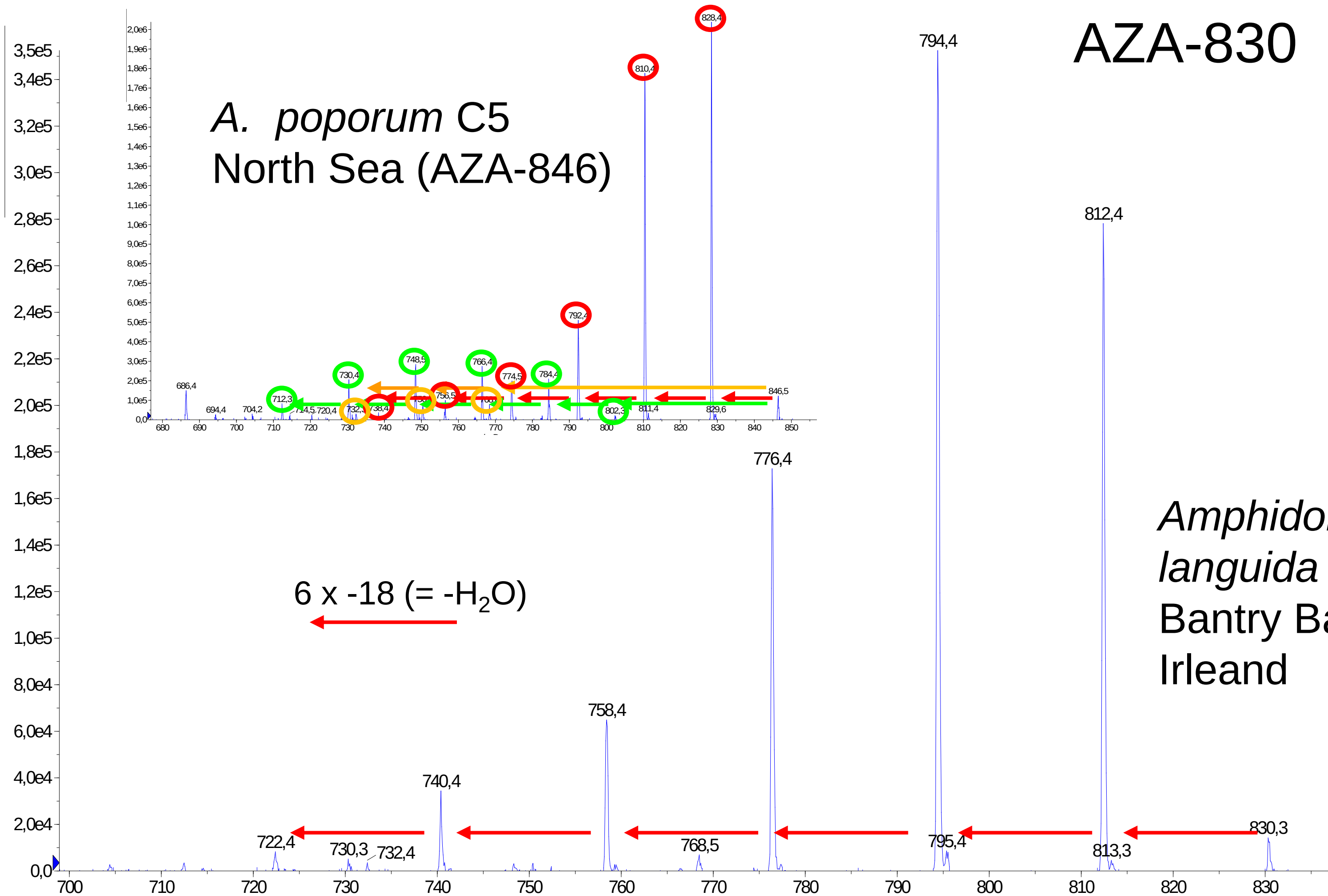

Amphidoma languida sp. nov., Bantry Bay, Irleand

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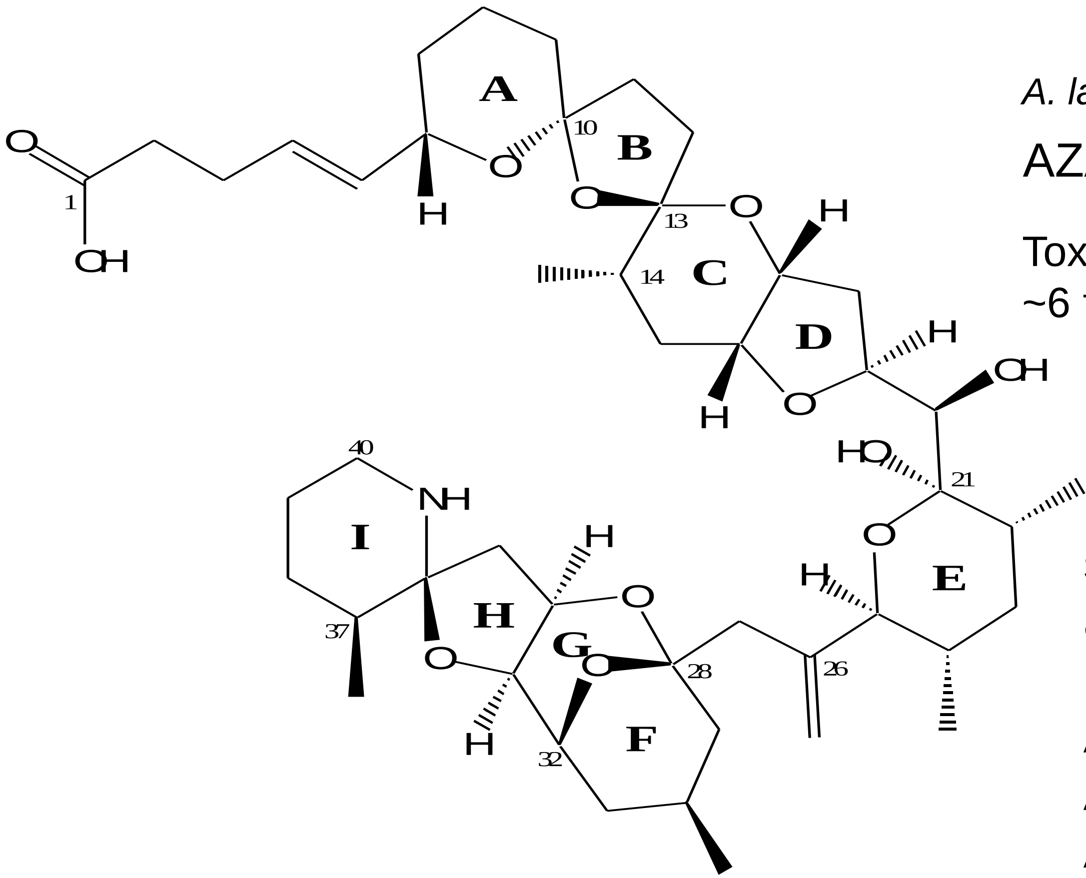
2. Novel toxins



A. languida:

AZA-830

Toxin cell quota:
~6 fg/cell

sum formulas
as determined by HRMS:

AZA-1:	$C_{47}H_{71}NO_{12}$
AZA-846:	$C_{46}H_{71}NO_{13}$
AZA-830:	$C_{46}H_{71}NO_{12}$

AZA-830 = 39-desmethyl-7,8-dihydro-AZA-1

hypothesized structure!

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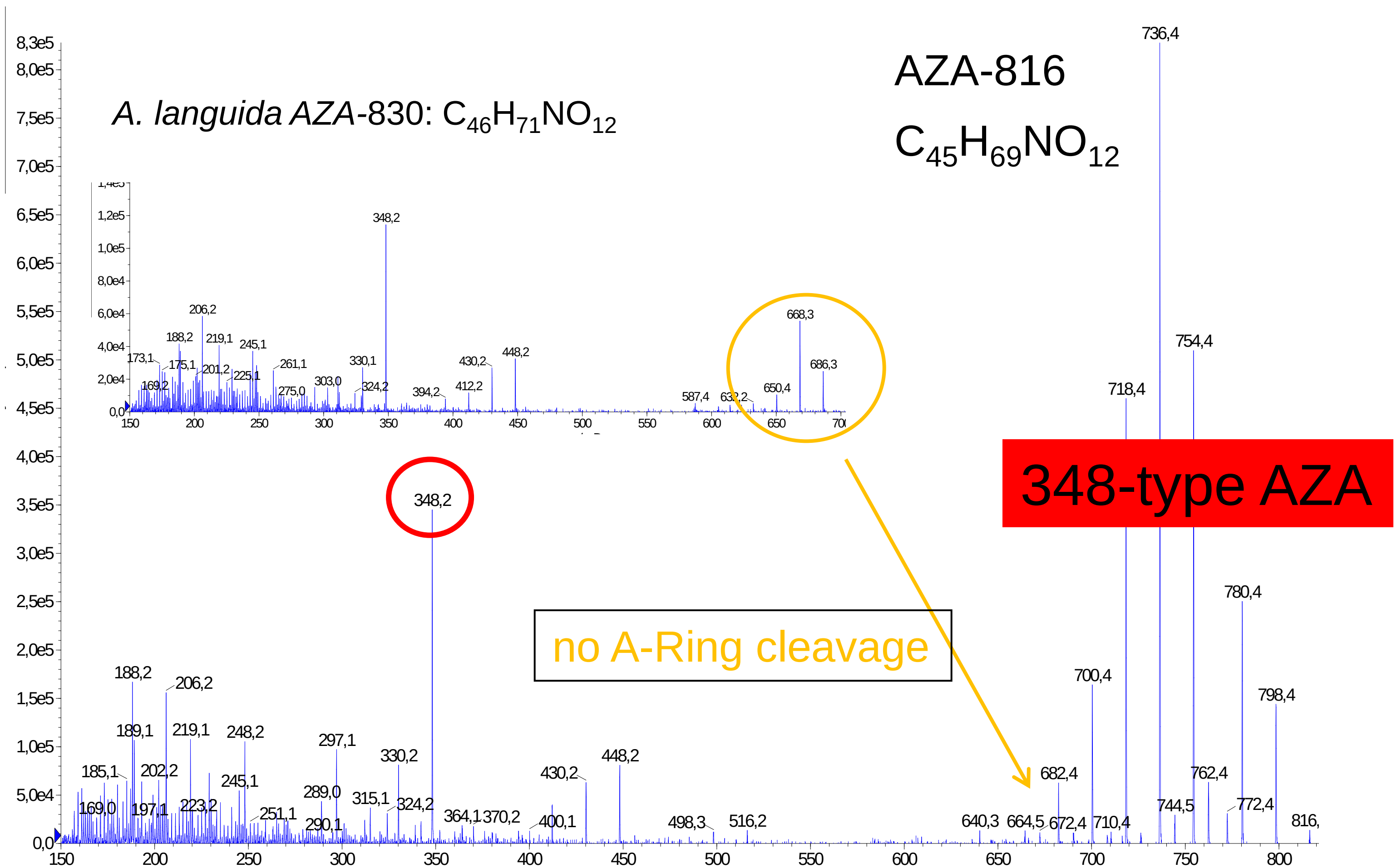
2. Novel toxins



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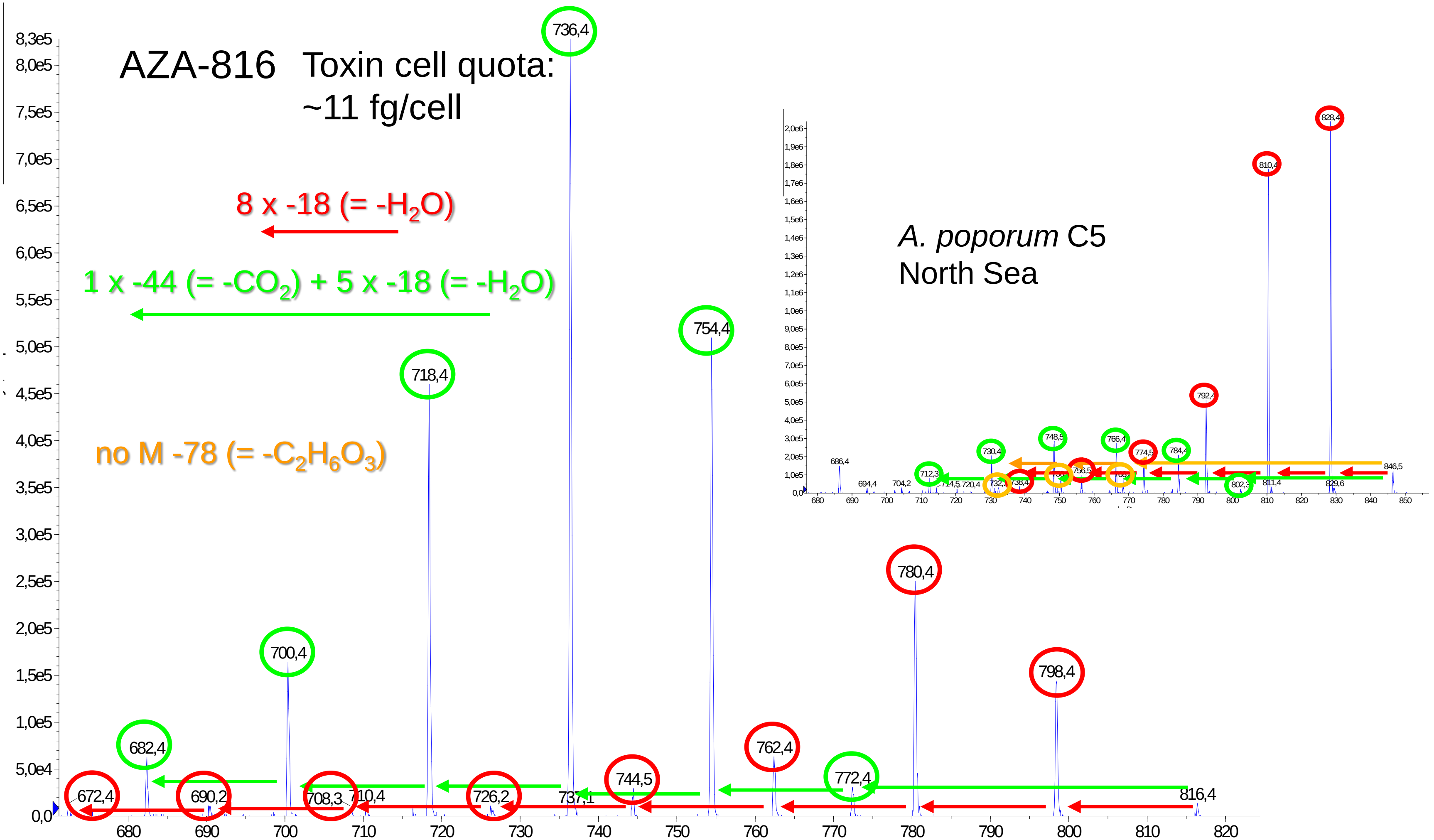
2. Novel toxins



Structure: **?**

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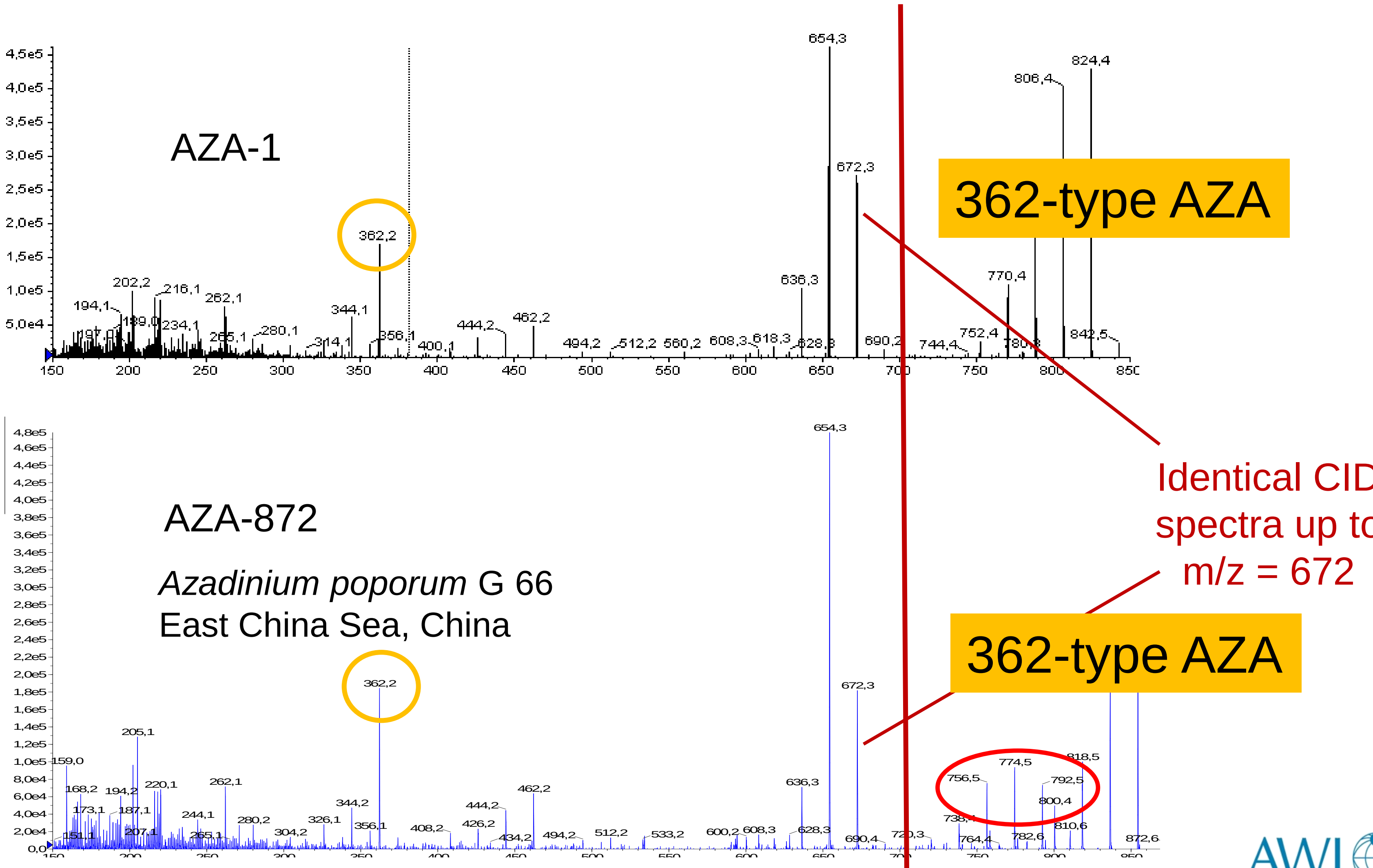
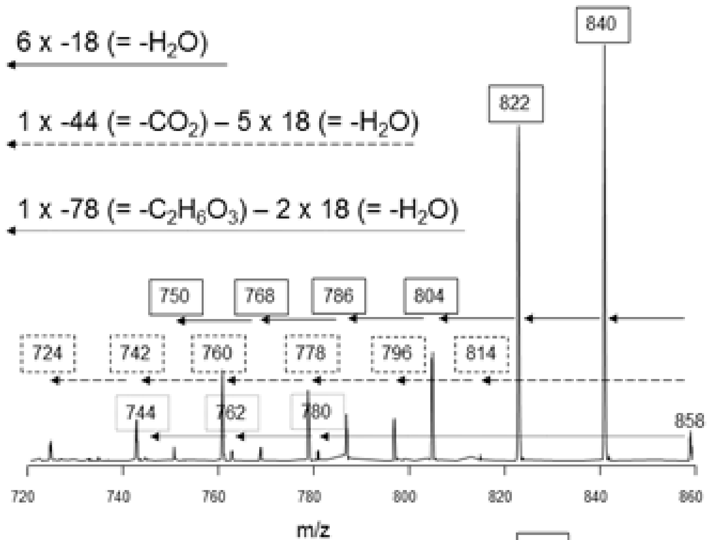
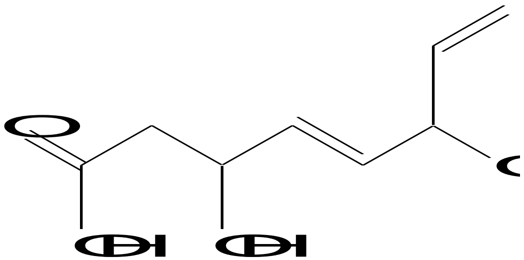
2. Novel toxins



2. Novel toxins

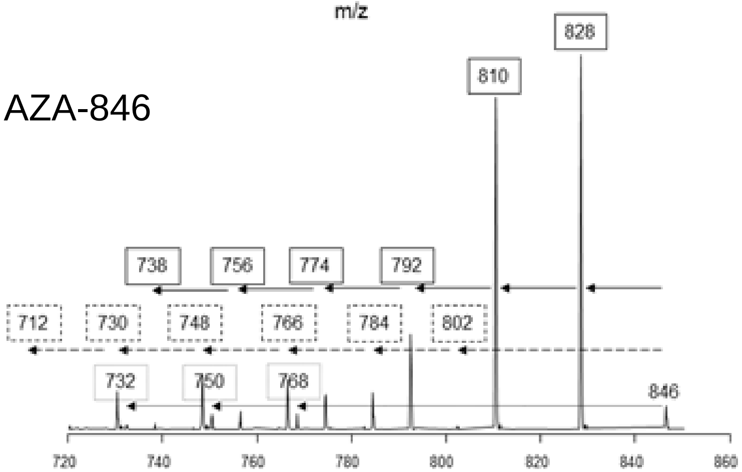
AZA-858



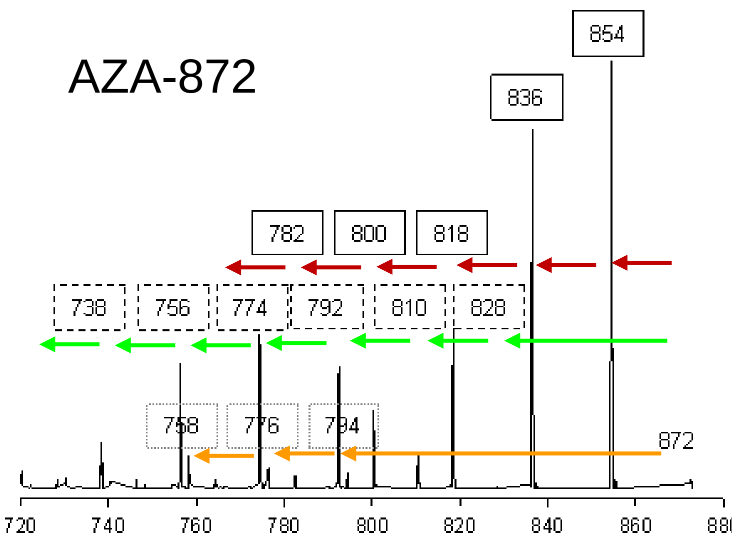


3-hydroxy-AZA

AZA-846



AZA-872



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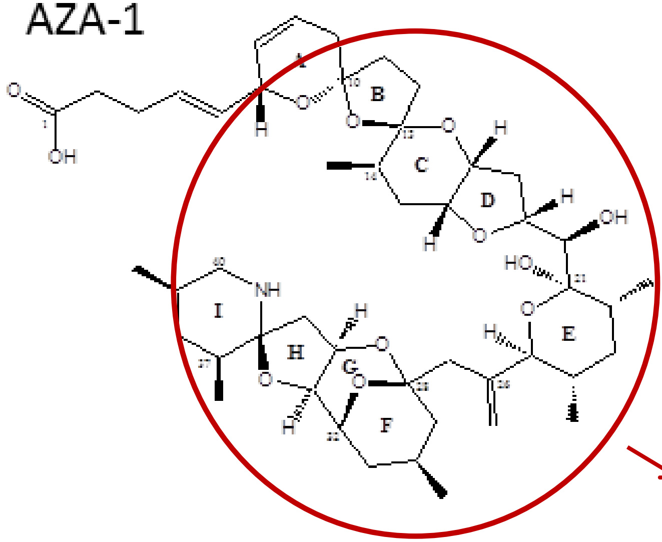
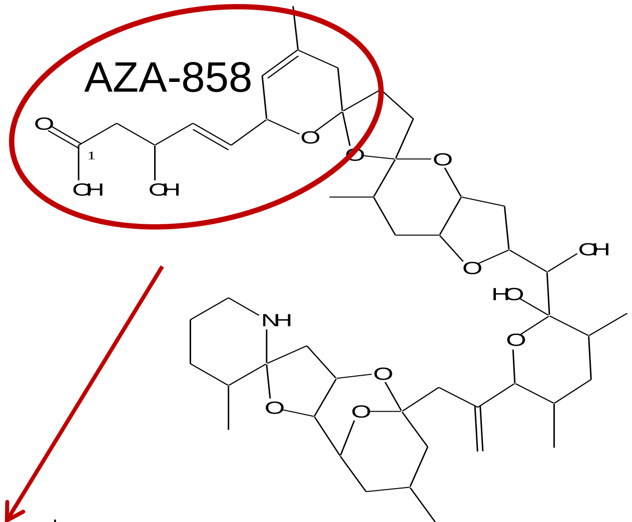
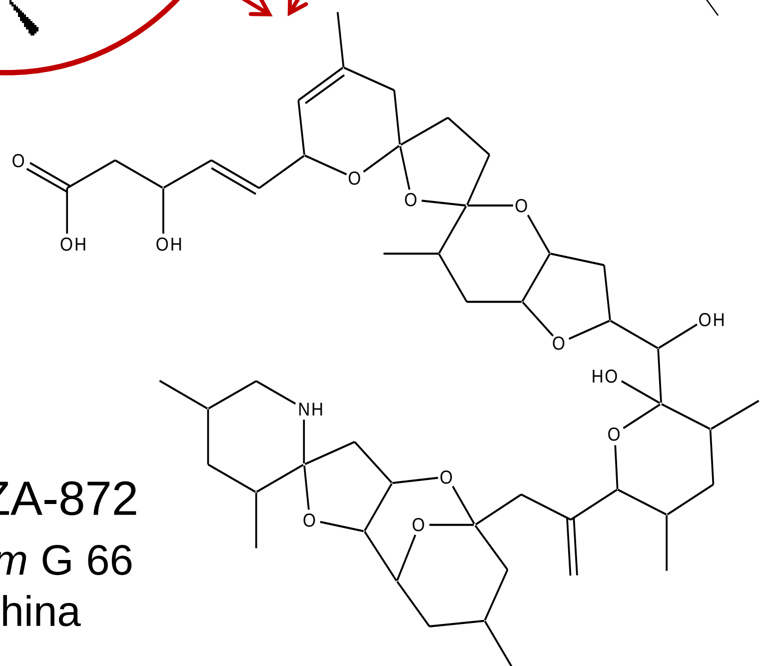
2. Novel toxins

AZA-1







AZA-872

Azadinium poporum G 66

East China Sea, China

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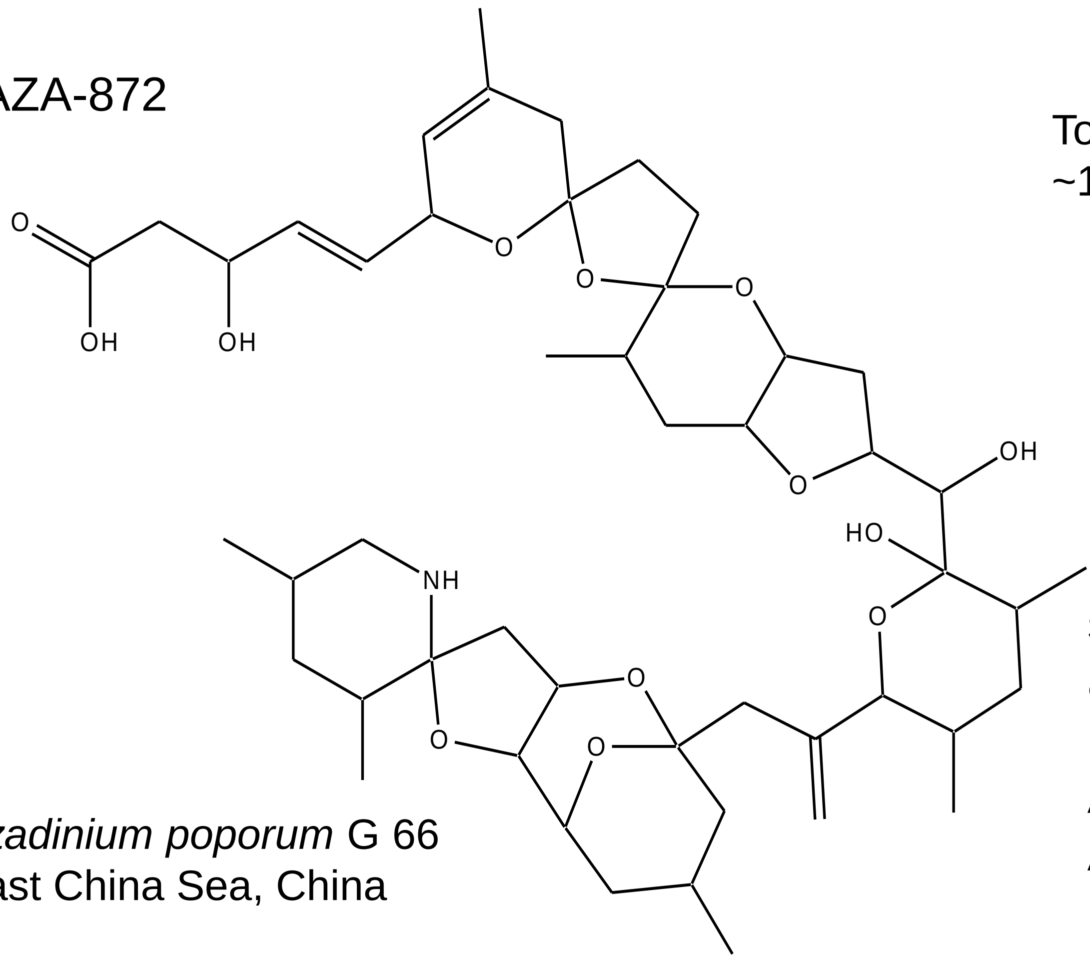
2. Novel toxins

AZA-872



Toxin cell quota:
~1.5 fg/cell

Azadinium poporum G 66
East China Sea, China

sum formulas
as determined by HRMS:

AZA-1: $C_{47}H_{71}NO_{12}$
AZA-872: $C_{48}H_{73}NO_{13}$

AZA-872 = 3-hydroxy-8-methyl-AZA-1 = AZA-11

confirmed by retention time and CID spectra comparison

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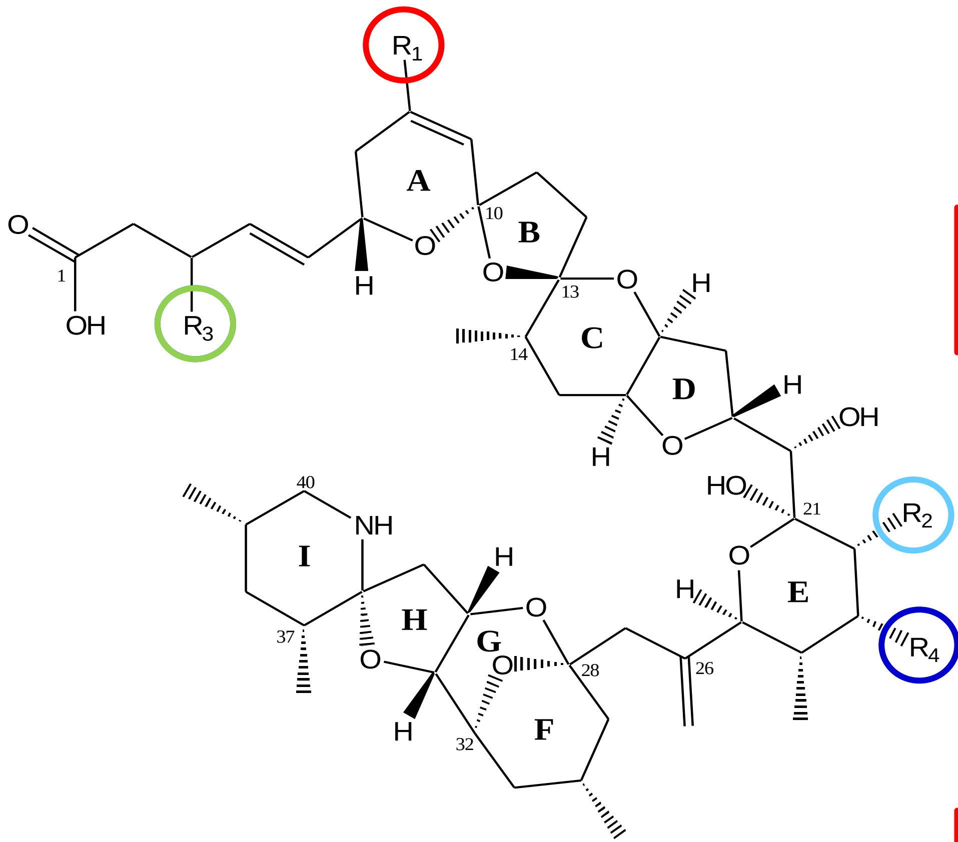
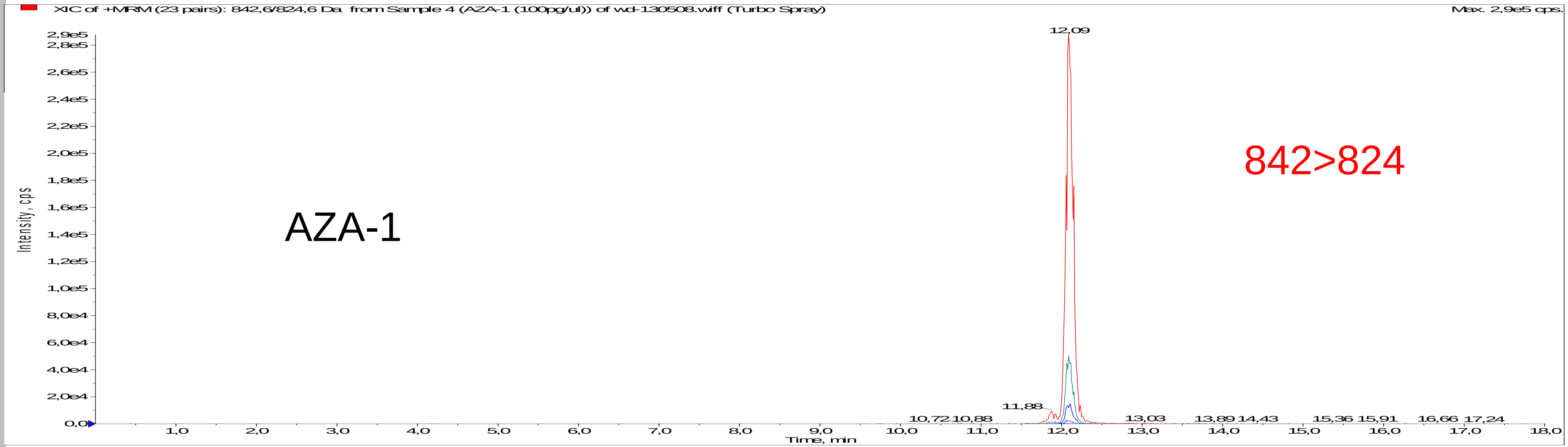


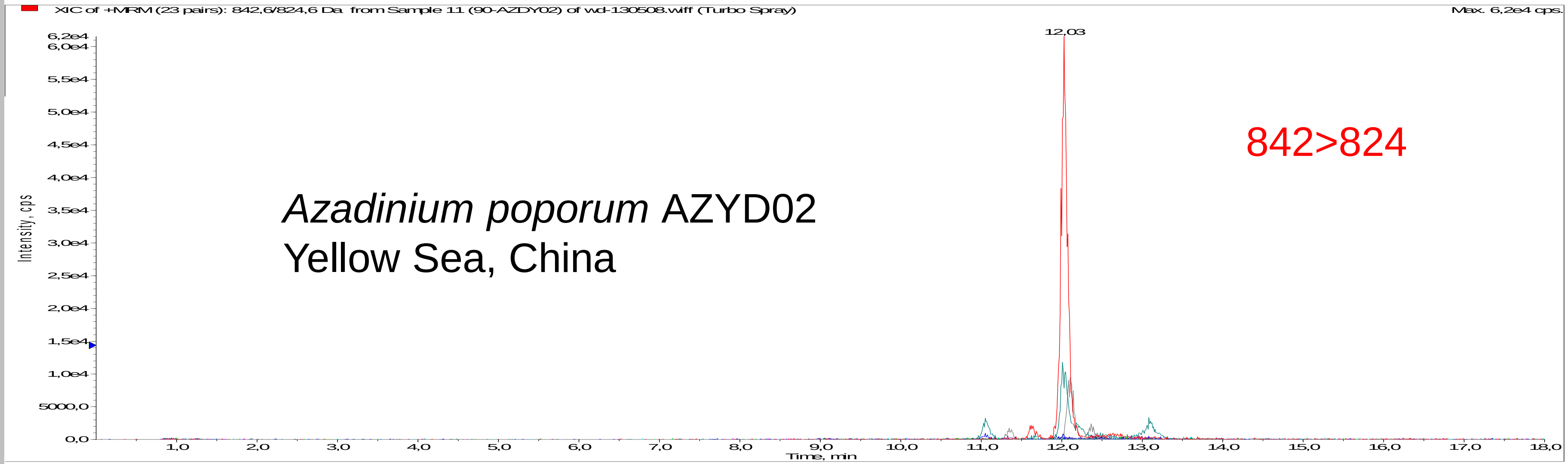
AZA-11

Toxin	R_1	R_2	R_3	R_4	$[M+H]^+$
AZA-1	H	CH_3	H	H	842
AZA-2	CH_3	CH_3	H	H	856
AZA-3	H	H	H	H	828
AZA-4	H	H	OH	H	844
AZA-5	H	H	H	OH	844
AZA-6	CH_3	H	H	H	842
AZA-7	H	CH_3	OH	H	858
AZA-8	H	CH_3	H	OH	858
AZA-9	CH_3	H	OH	H	858
AZA-10	CH_3	H	H	OH	858
AZA-11	CH_3	CH_3	OH	H	872

(Krock et al. in preparation)

3. Novel toxins





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2. Novel toxins

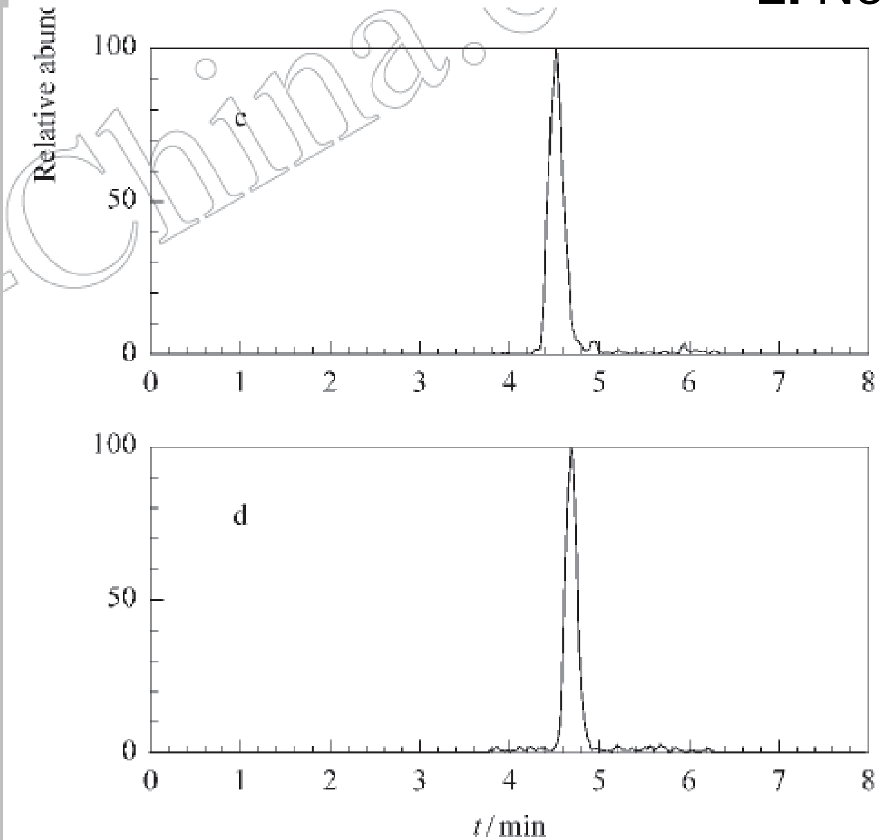


图 3 (a) AZA1 标准溶液(488. 5 ng/L)、(b)空白扇贝肌肉、(c)加标扇贝肌肉(73. 27 pg/g)和(d)栉孔扇贝阳性样品的色谱图

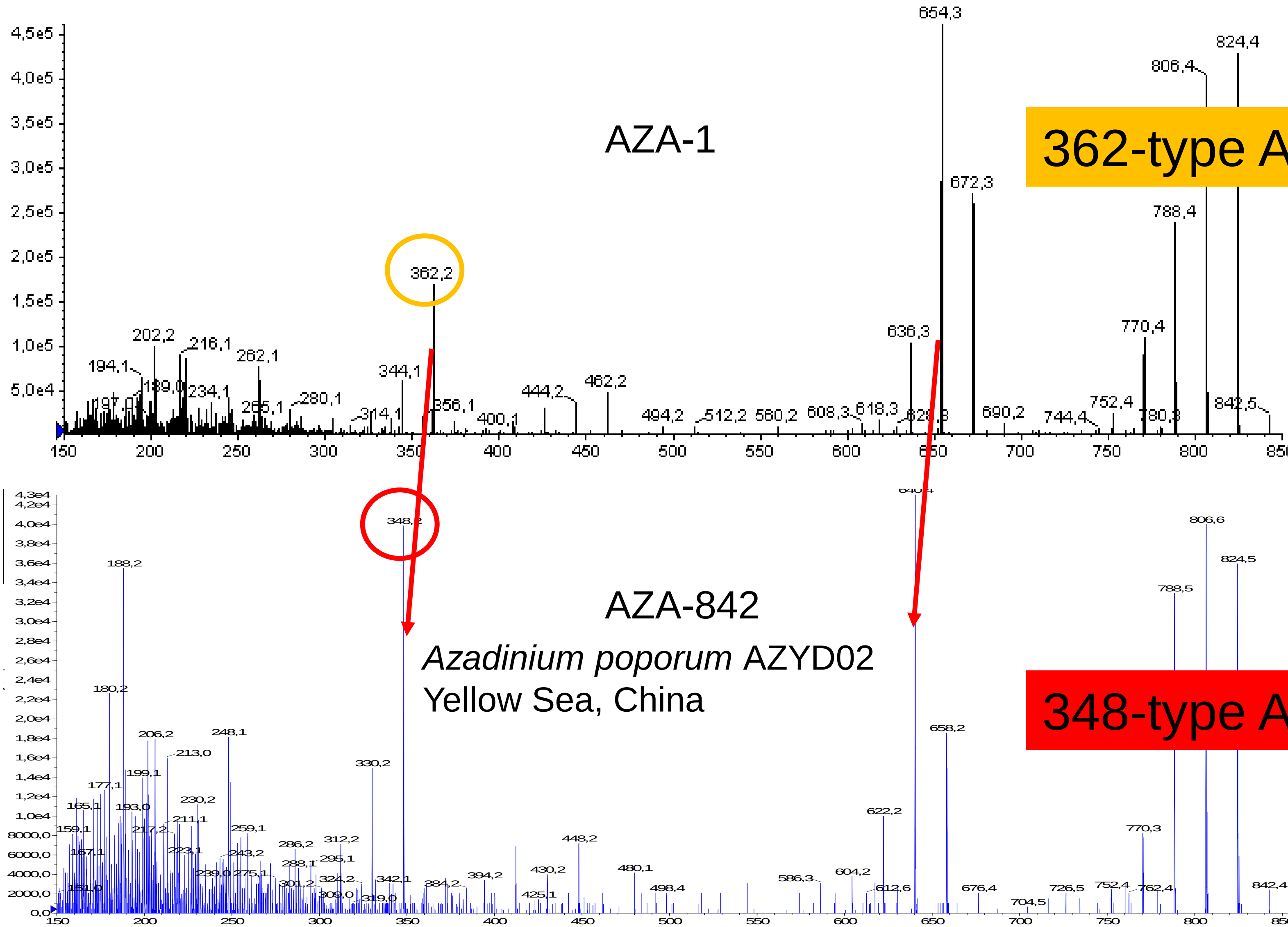
Fig. 3 Chromatograms of (a) a standard solution of AZA1 (488. 5 ng/L), (b) a blank scallop muscle sample, (c) a blank scallop muscle sample spiked with AZA1 of 73. 27 pg/g and (d) a polluted scallop muscle sample

Yao J., Tan Z., Zhou D., Guo M., Xing L., Yang S., 2010:

Determination of azaspiracid-1 in shellfish by liquid chromatography with tandem mass spectrometry.

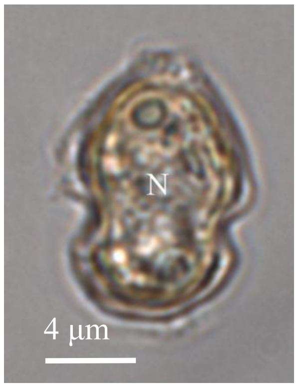
Chinese Journal of Chromatography 28, 363-367.

2. Novel toxins

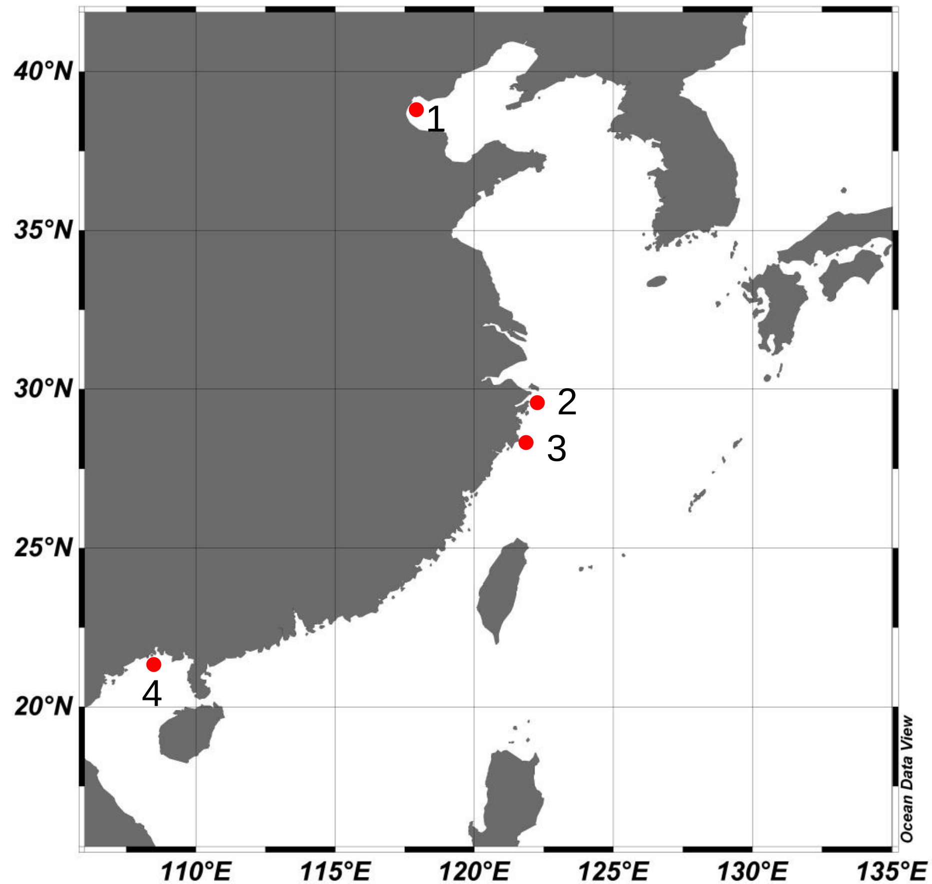


(Krock et al. in preparation)

3. Geographic distribution



Azadinium poporum, China



Gu et al. 2013, *Harmful Algae* 21–22, 64-75.

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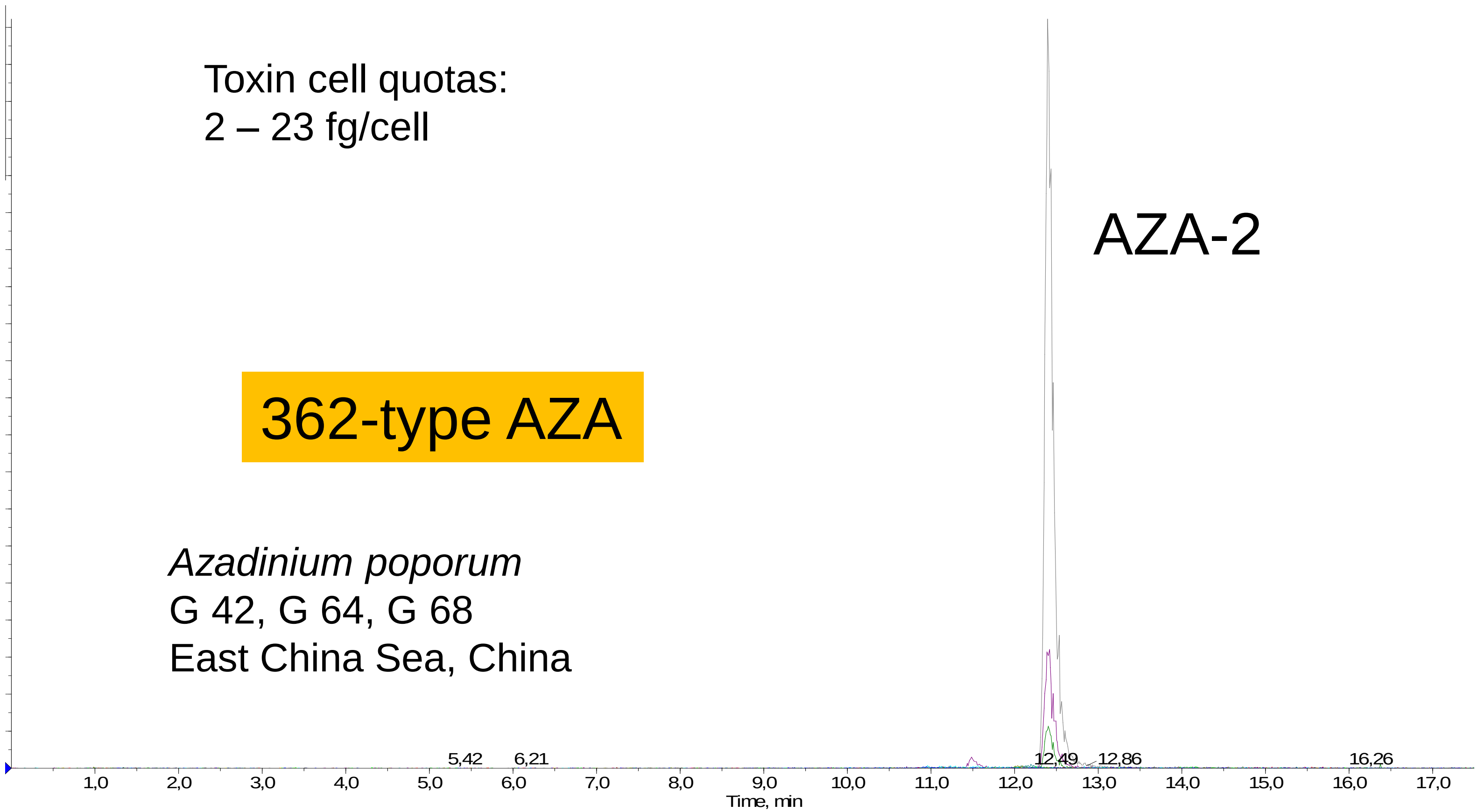

3. Geographic distribution

Toxicon 53 (2009) 680–684

Contents lists available at ScienceDirect

Toxicon

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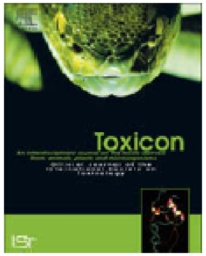

ELSEVIER

Isolation of azaspiracid-2 from a marine sponge *Echinoclathria* sp. as a potent cytotoxin

Reiko Ueoka [a], Akihiro Ito [b], Miho Izumikawa [c], Satoko Maeda [b], Motoki Takagi [c], Kazuo Shin-ya [c], Minoru Yoshida [b], Rob. W.M. van Soest [d], Shigeki Matsunaga [a,*]

[a] *Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan*
[b] *Chemical Genomics Research Group, RIKEN, Wako, Saitama 351-0198, Japan*
[c] *Biomedicinal Information Research Center, National Institute of Advanced Industrial Science and Technology, Koto-ku, Tokyo 135-0064, Japan*
[d] *Zoological Museum, University of Amsterdam, 1090GT Amsterdam, The Netherlands*

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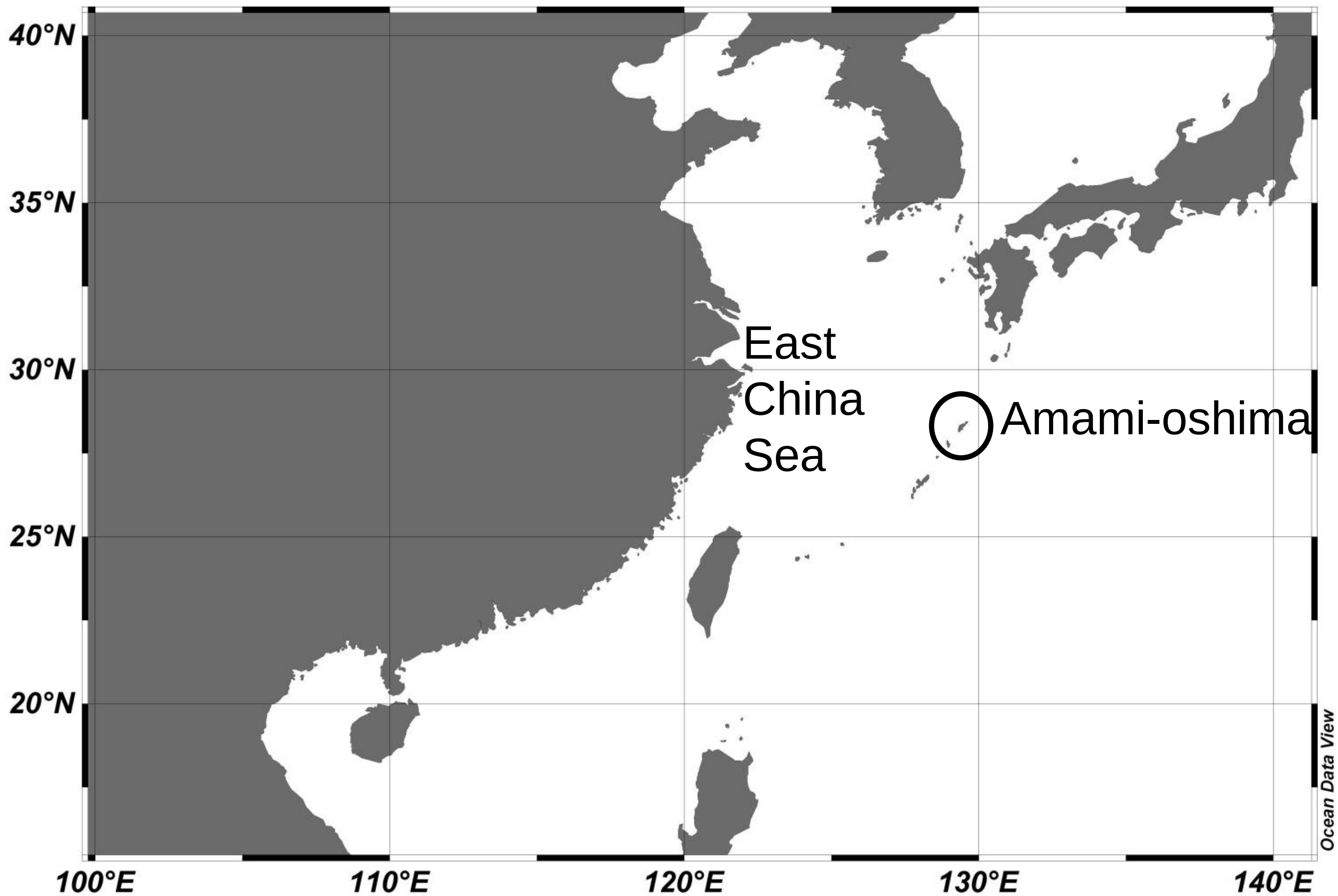
3. Geographic distribution

A. pororum strains G 42, G 64 and G 68 are probably the source of the sponge contamination with AZA-2



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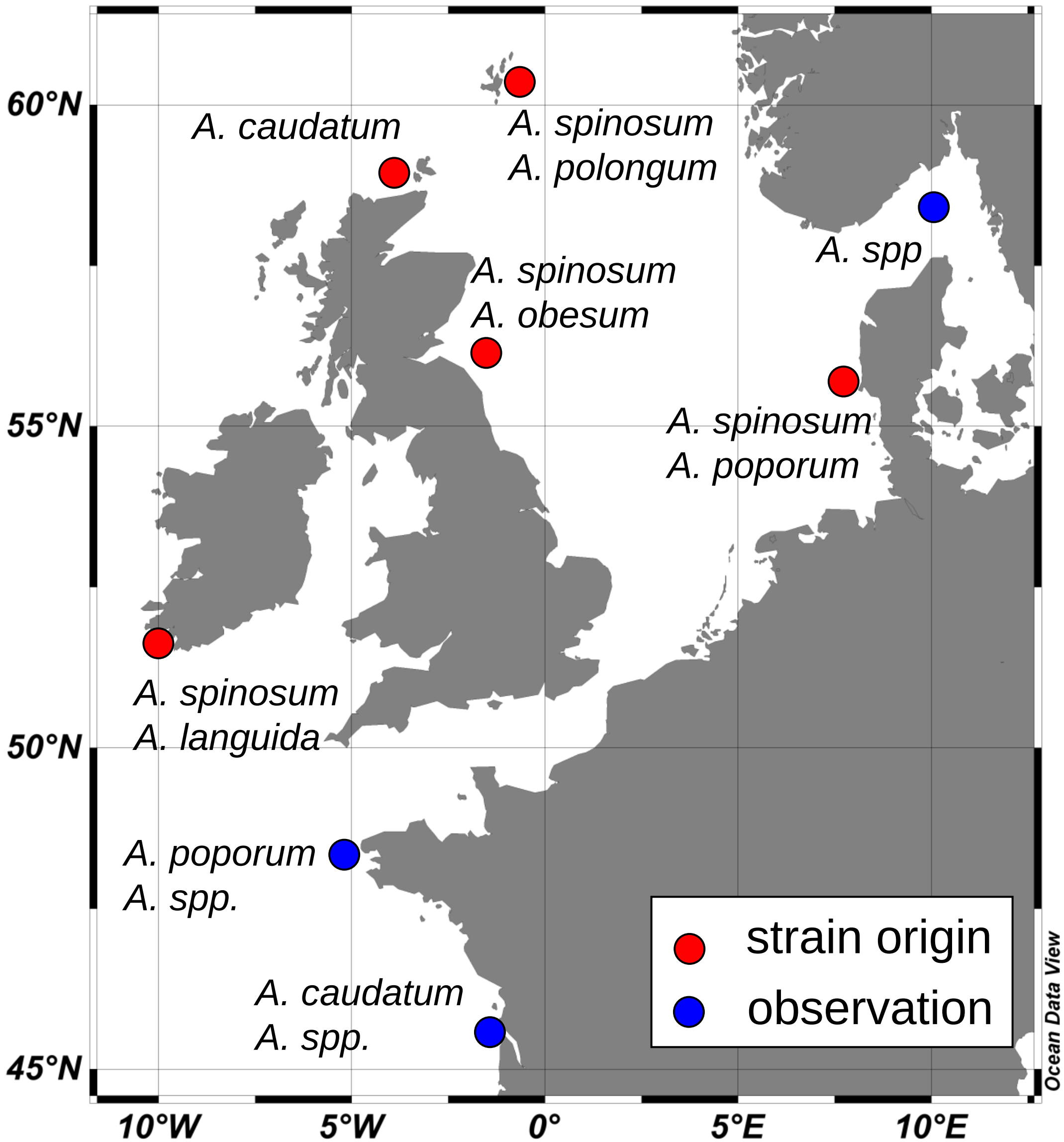

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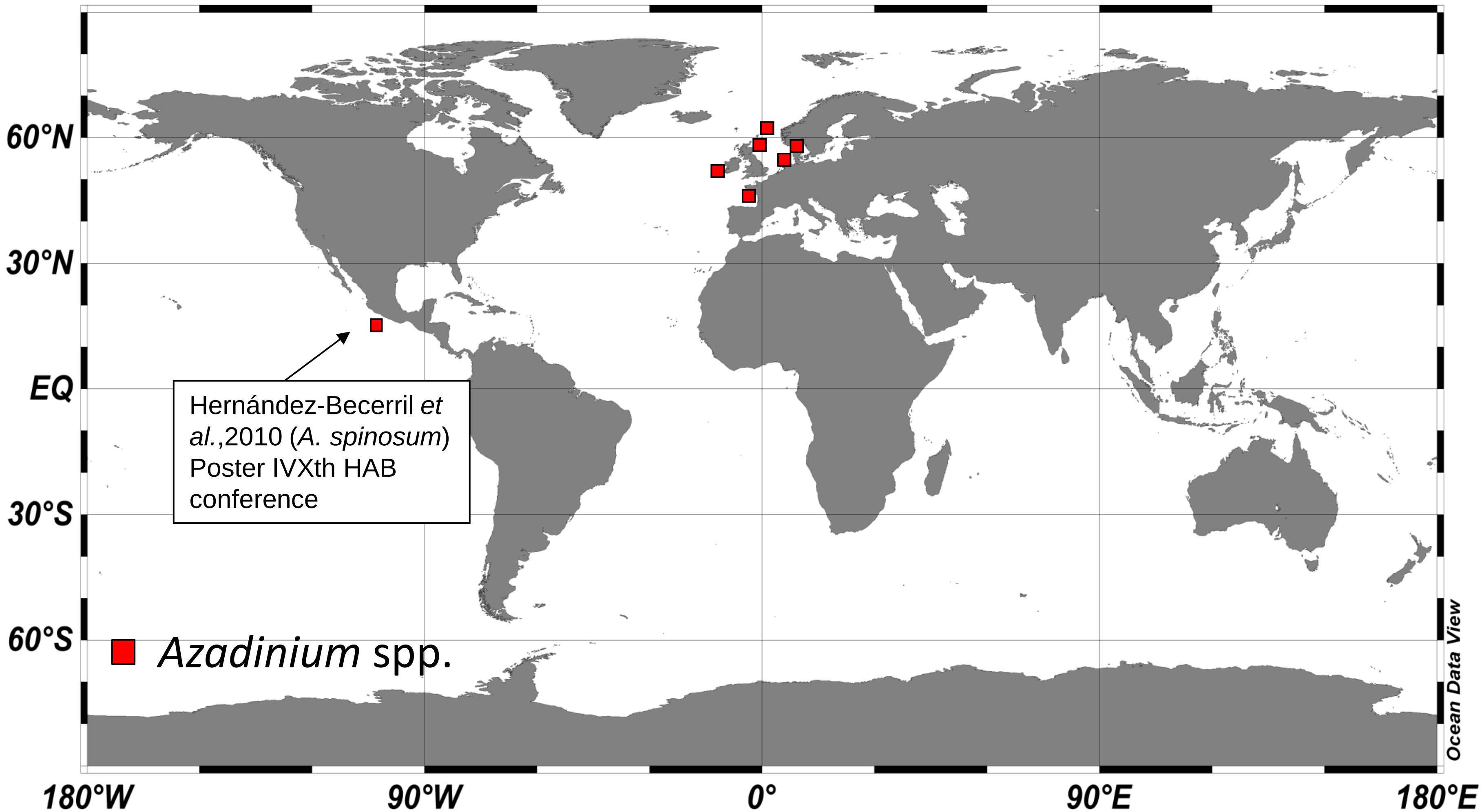

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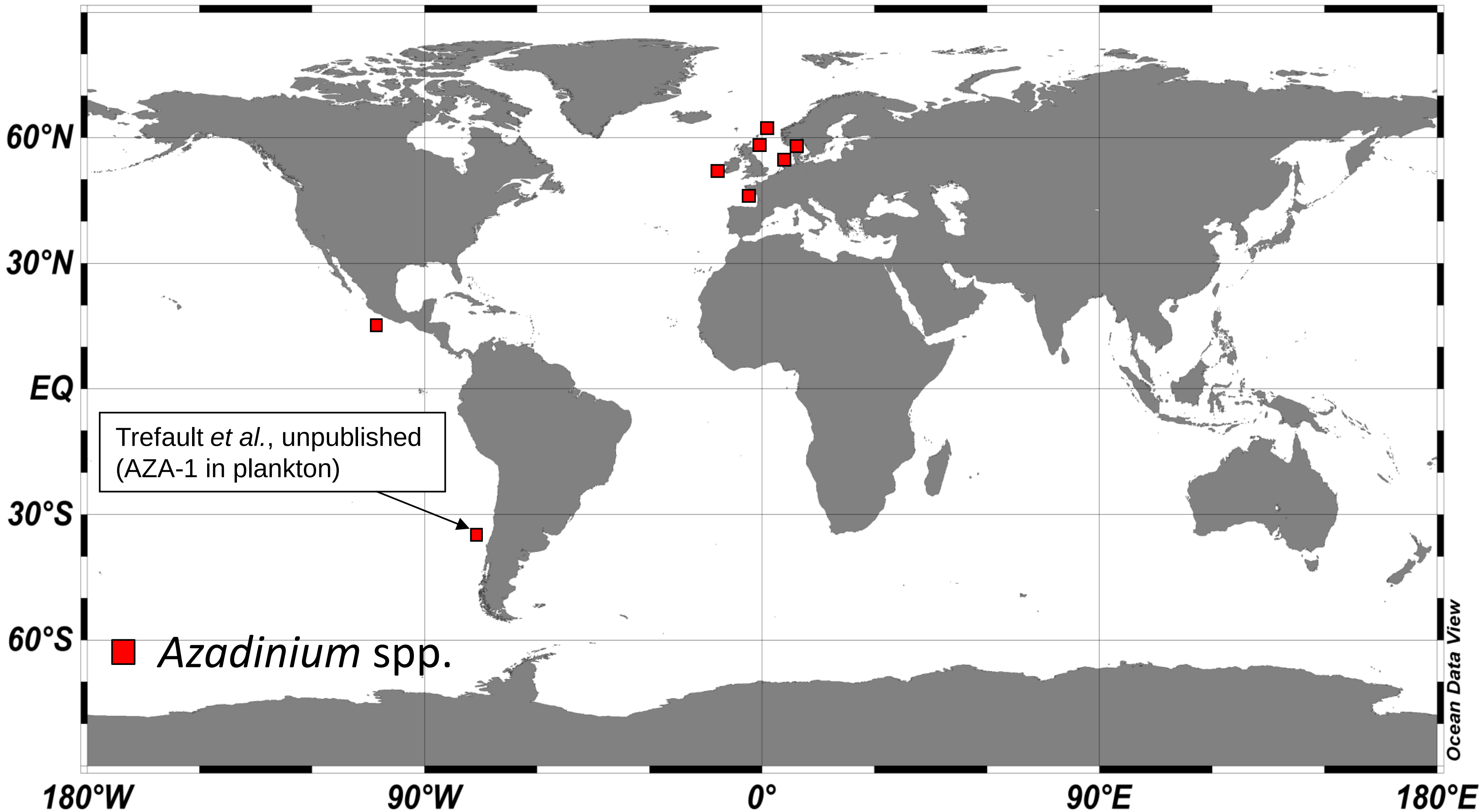
3. Geographic distribution



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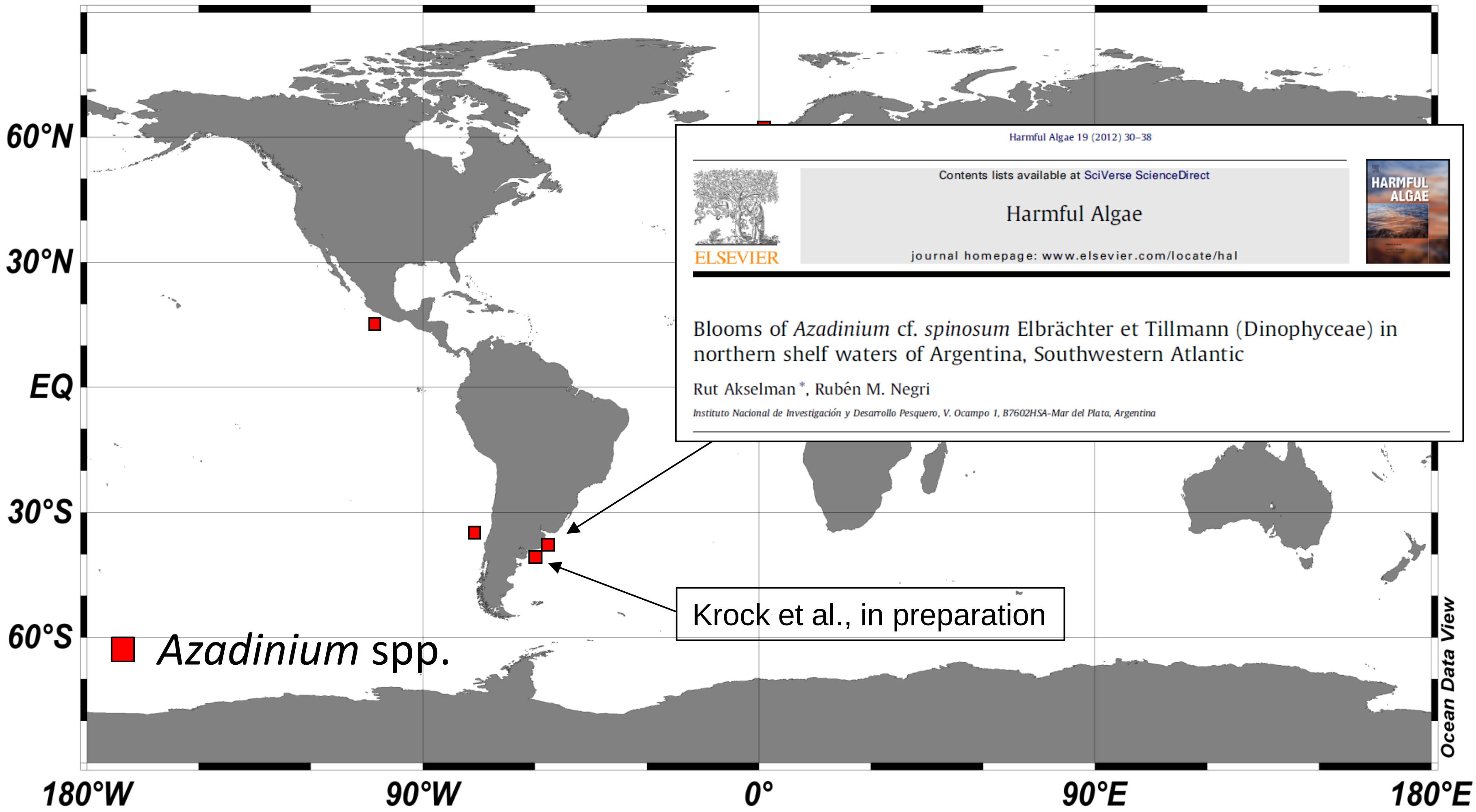
3. Geographic distribution



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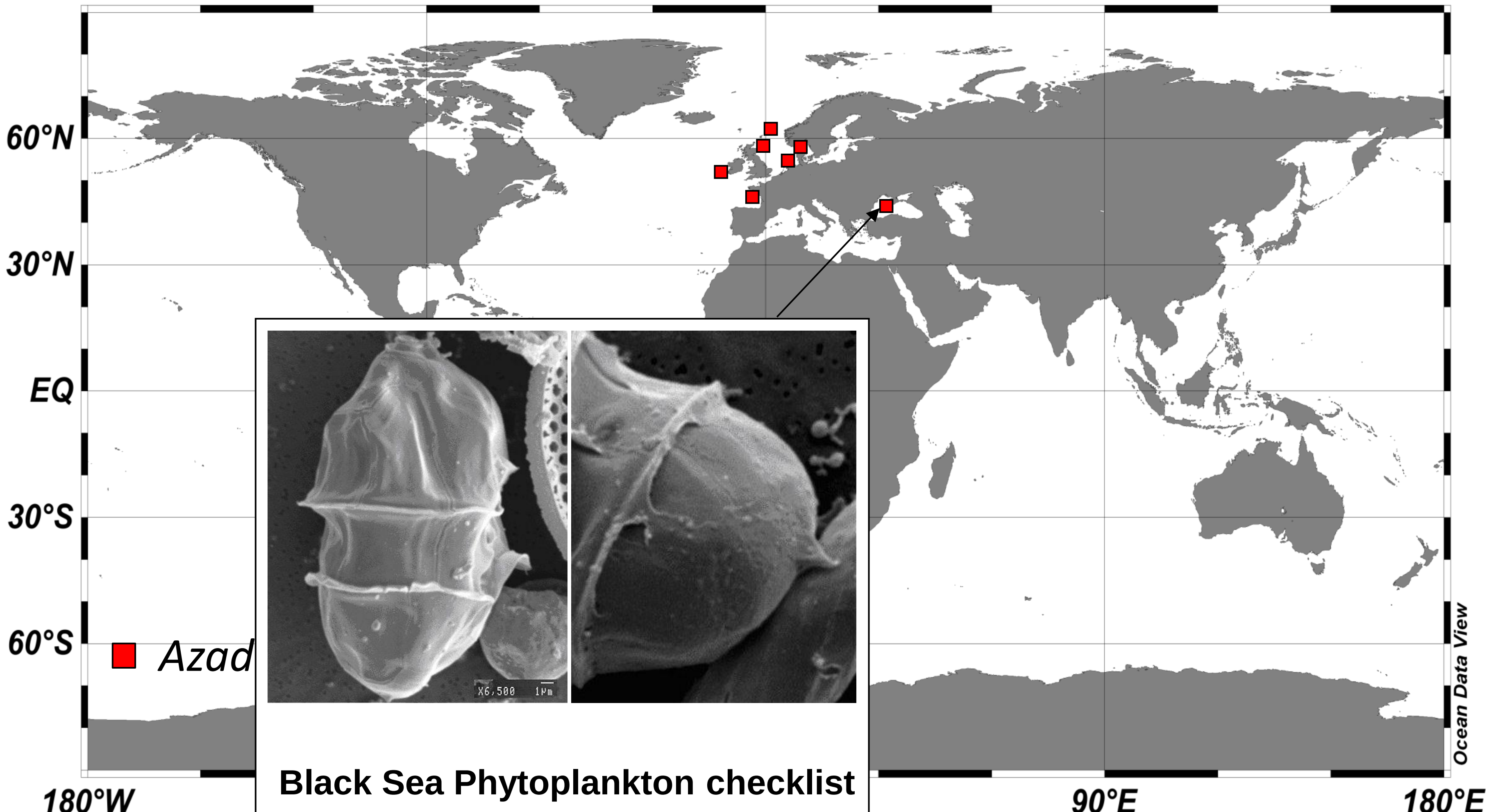
3. Geographic distribution



Black Sea Phytoplankton checklist

http://phyto.bss.ibss.org.ua/wiki/Azadinium_spinosum

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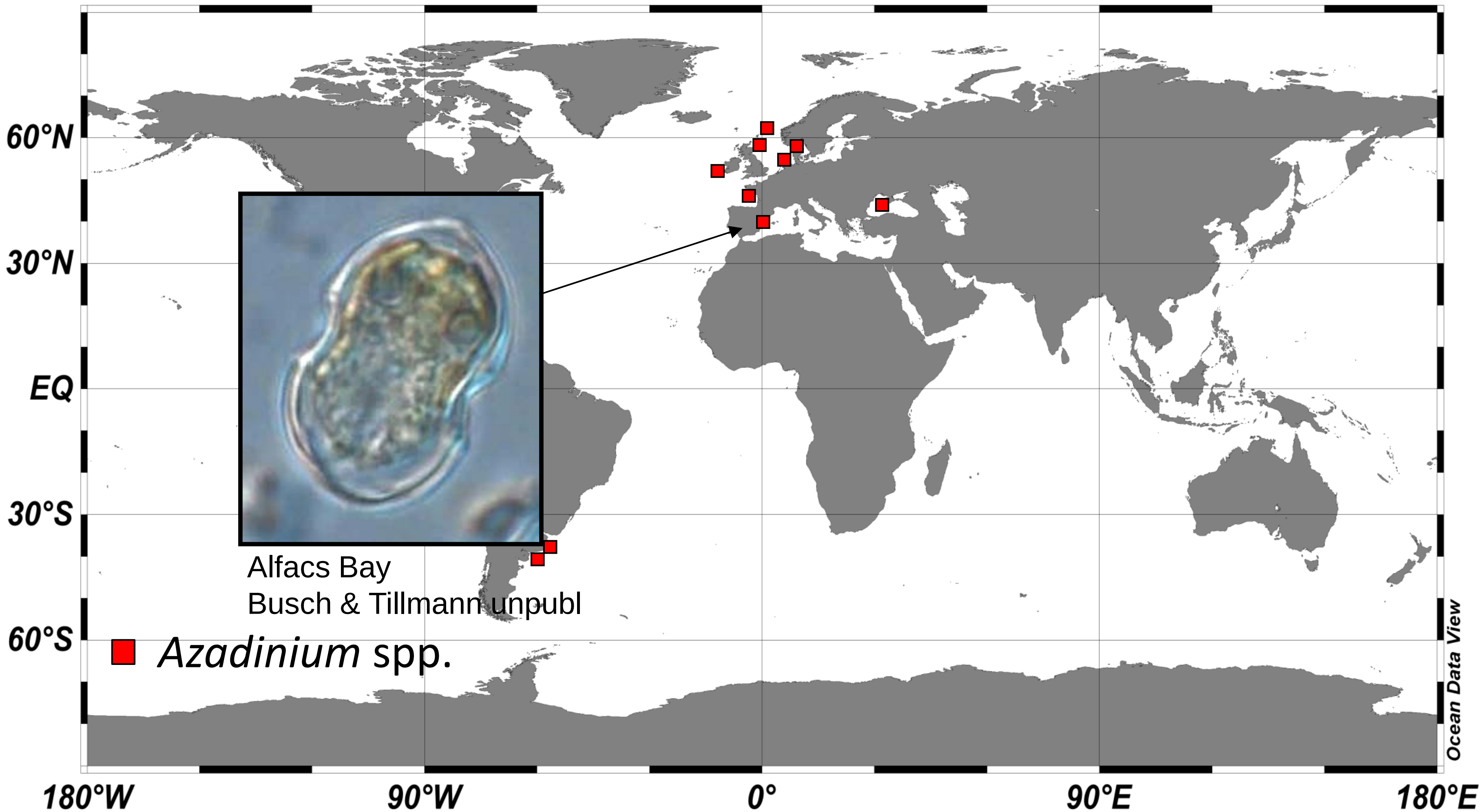
3. Geographic distribution



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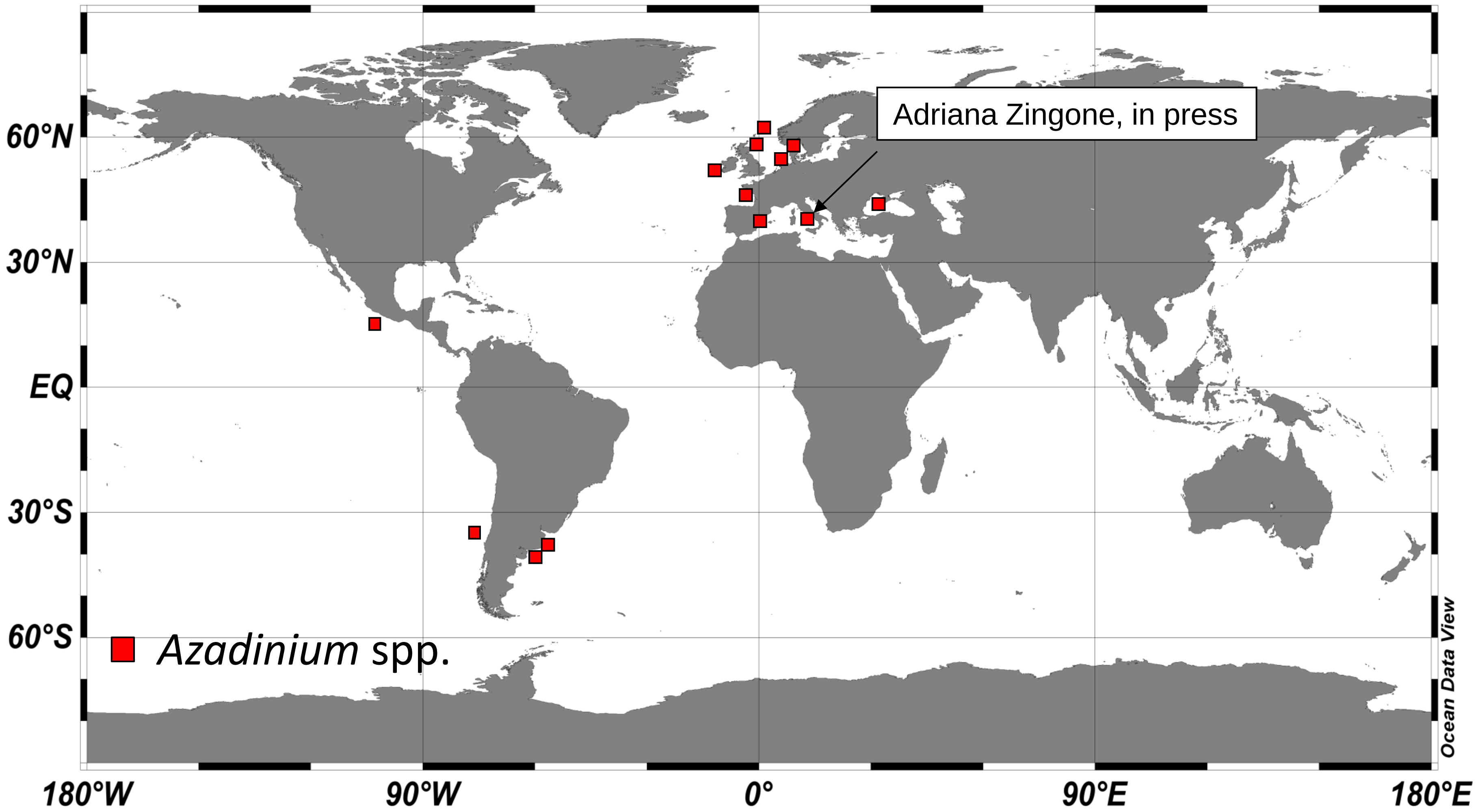
3. Geographic distribution



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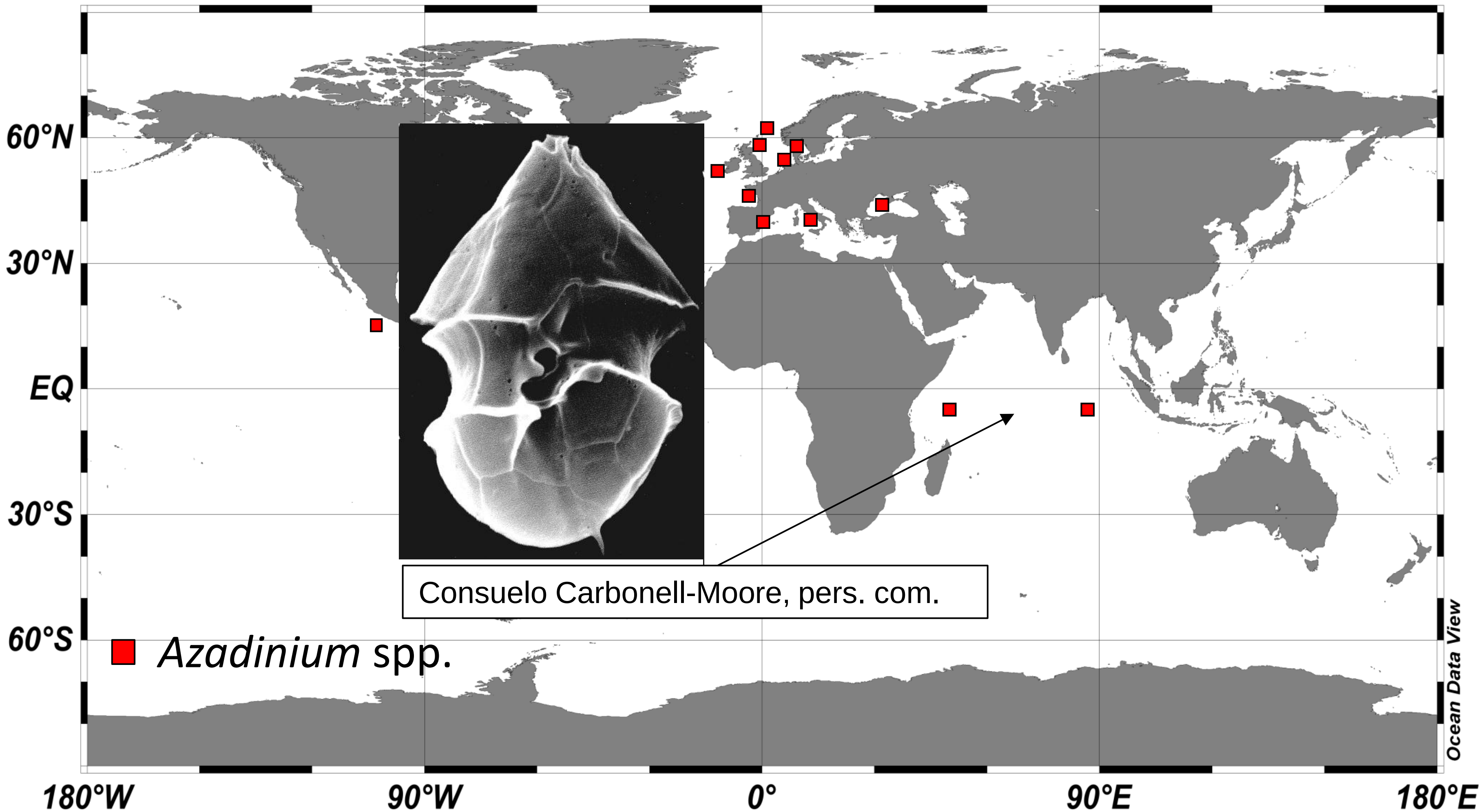
3. Geographic distribution



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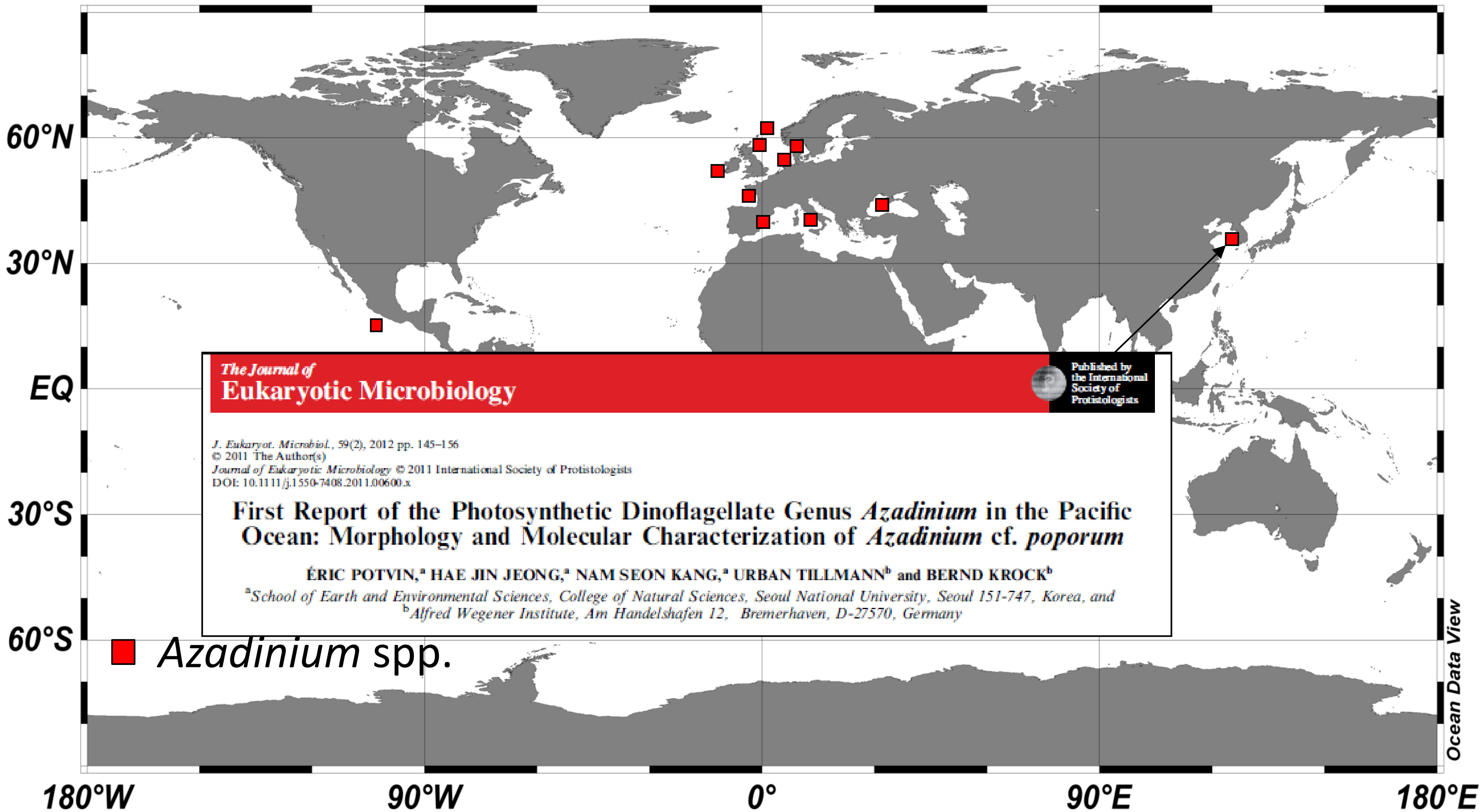

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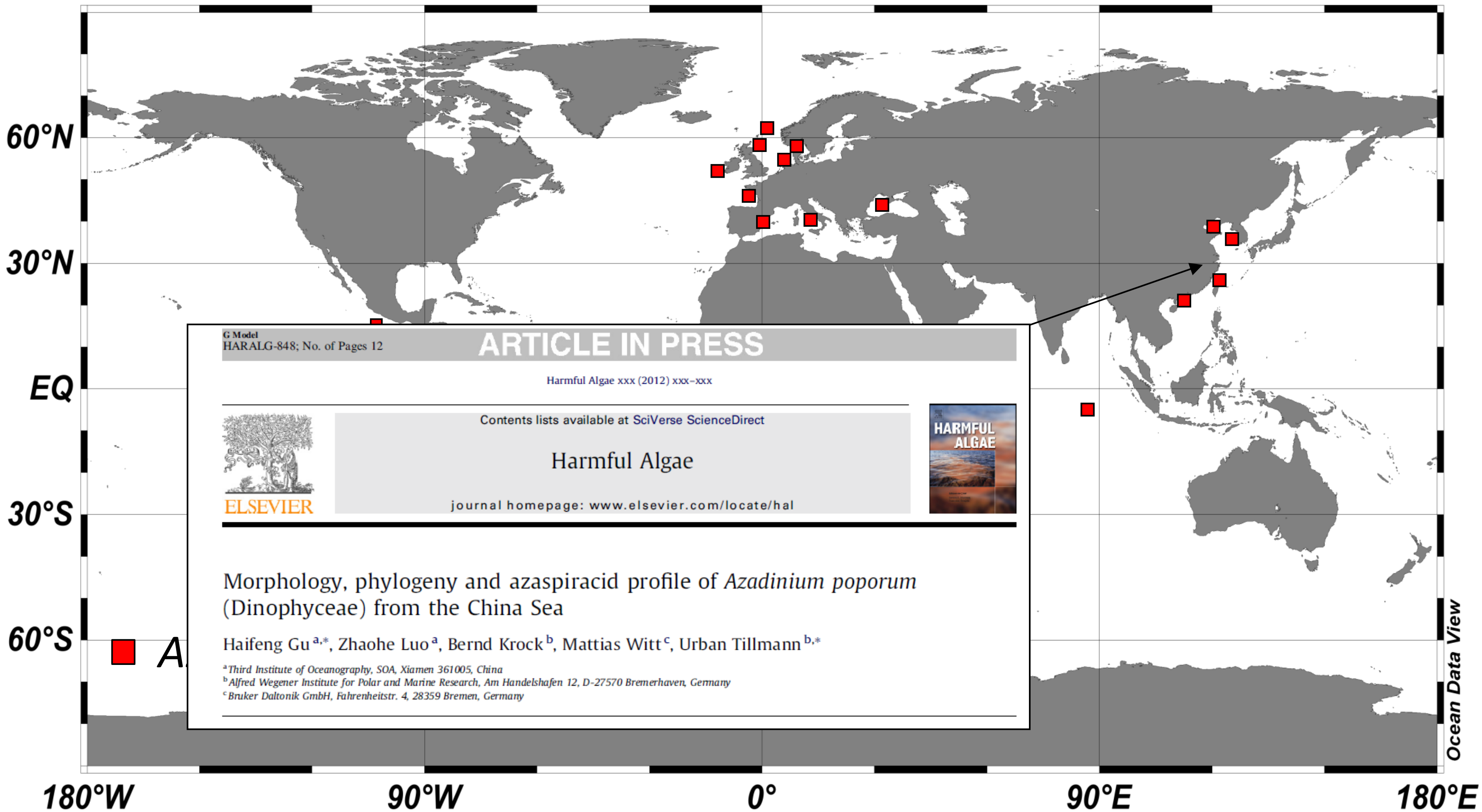

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Harmful Algae xxx (2012) xxx–xxx

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Harmful Algae

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

ELSEVIER

Morphology, phylogeny and azaspiracid profile of *Azadinium poporum* (Dinophyceae) from the China Sea

Haifeng Gu[a,*], Zhaohe Luo[a], Bernd Krock[b], Mattias Witt[c], Urban Tillmann[b,*]

[a] Third Institute of Oceanography, SOA, Xiamen 361005, China
[b] Alfred Wegener Institute for Polar and Marine Research, Am Handelshafen 12, D-27570 Bremerhaven, Germany
[c] Bruker Daltonik GmbH, Fahrenheitstr. 4, 28359 Bremen, Germany

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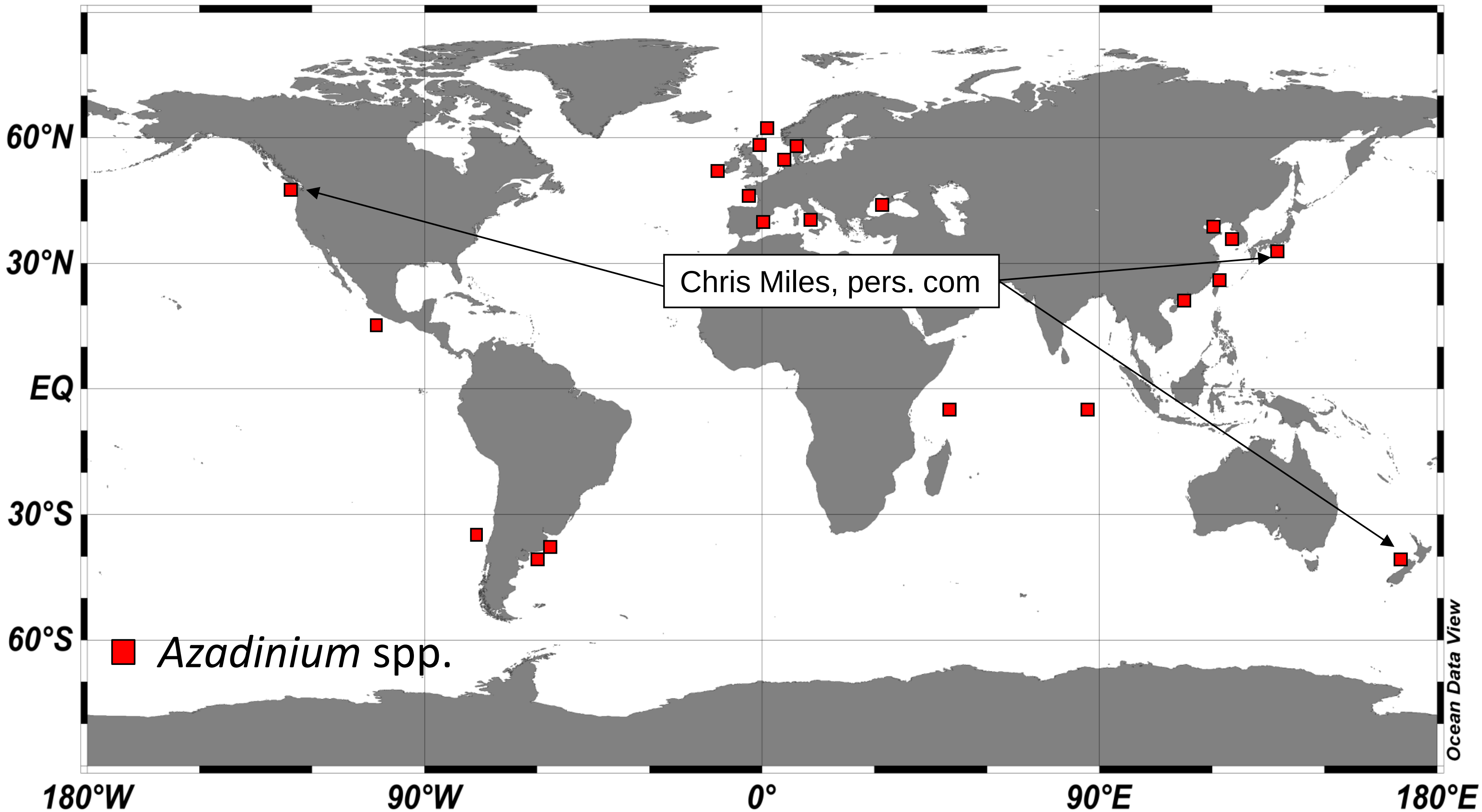

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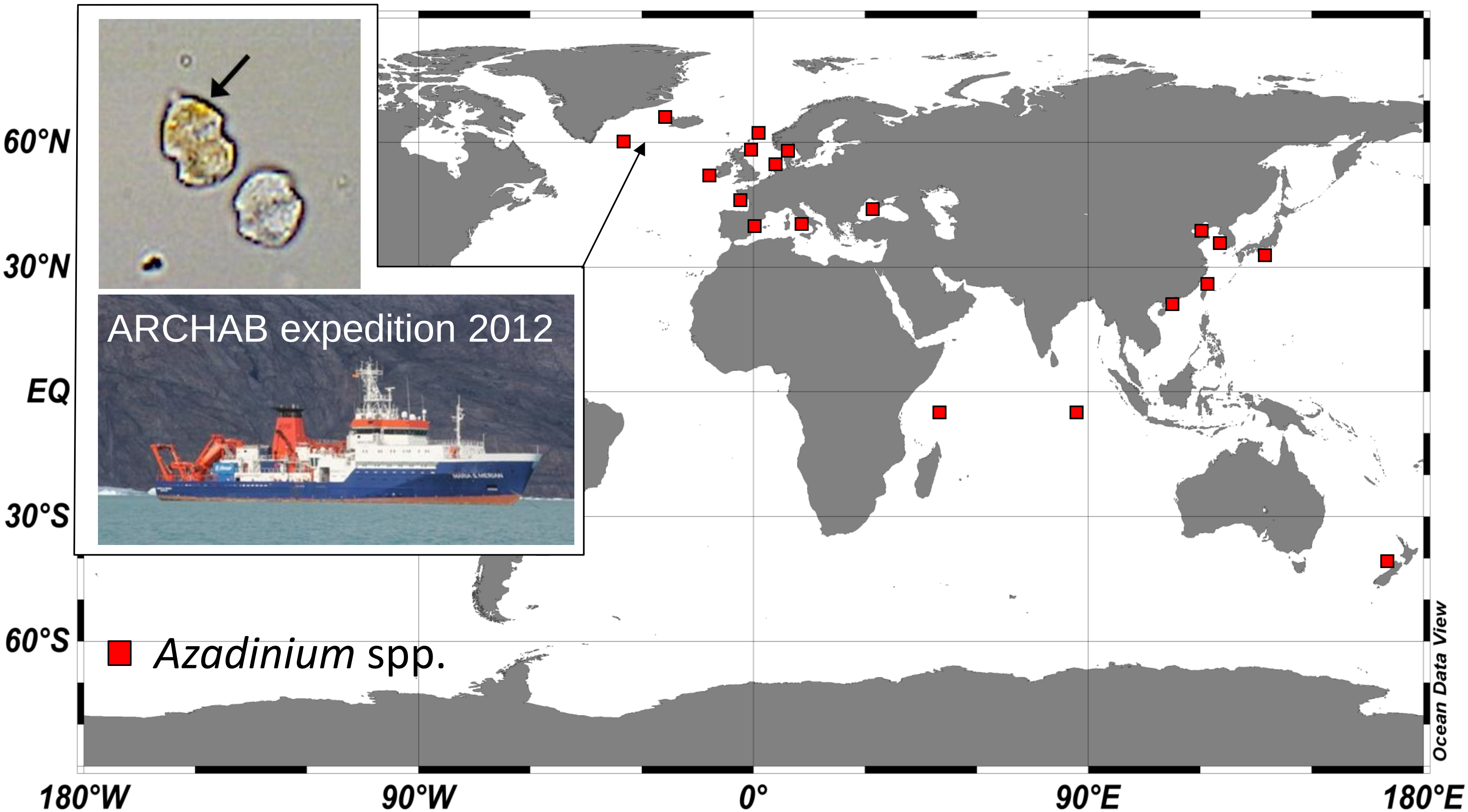

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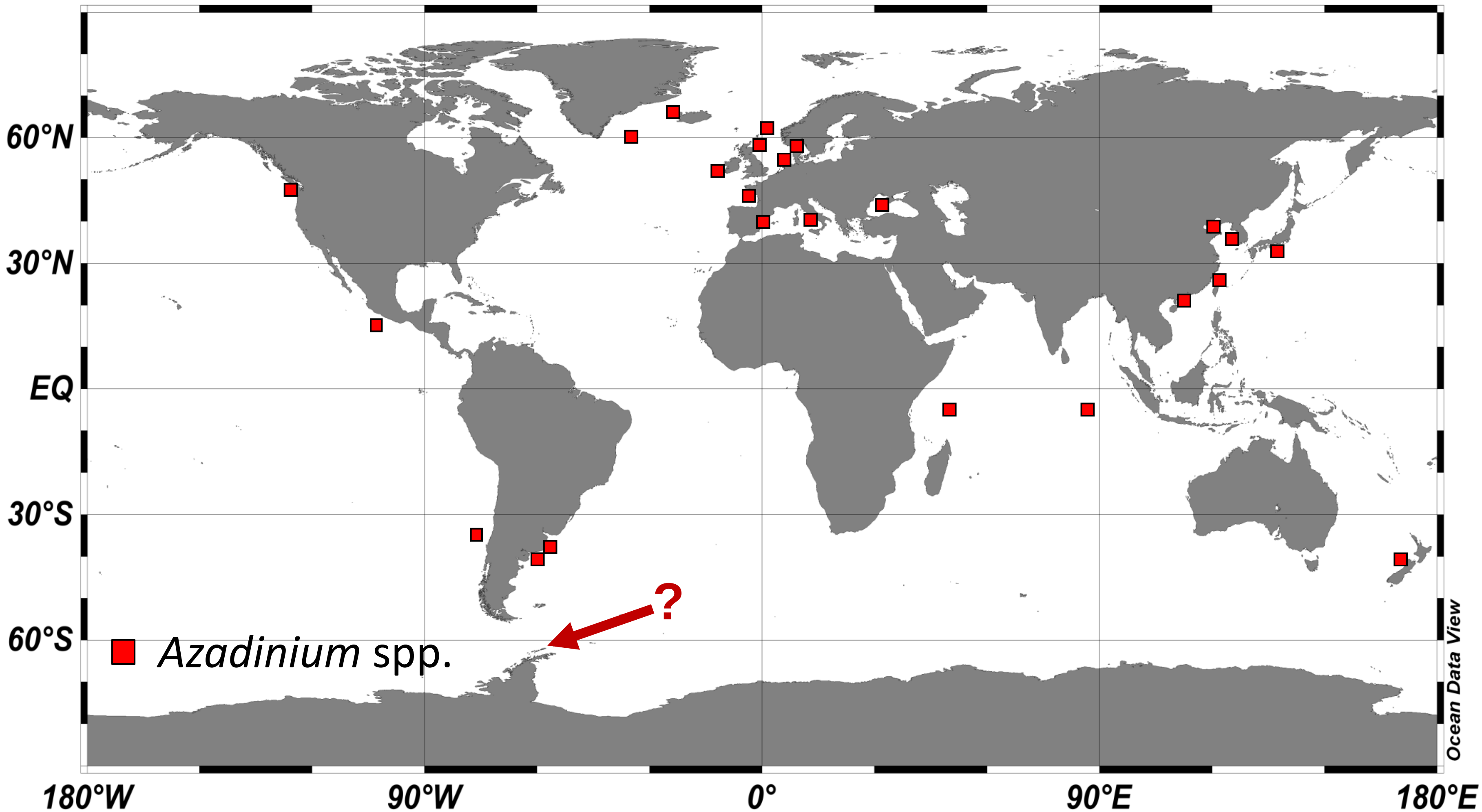

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4. Take home message

1. Since 2007 three species are known to be *de novo* producers of AZAs: *Azadinium spinosum*, *A. poporum*, *A. dexteroporum* and *Amphidoma languida*

2. Today more than 10 AZAs are known be produced by dinoflagellates: AZA-1, -2,-11, -33-41

3. The occurrence of Azadinum spp. and AZAs is a global problem and not restricted to northwest European waters

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Thanks to…

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Tilman Alpermann	Universität Oldenburg
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Rafael Salas	Marine Institute, IE
Elisabeth Nézan	IFREMER, FR
Philipp Hess	IFREMER, FR
Éric Potvin	Seoul University, KR
Hae Jin Jeong	Seoul University, KR
Haifeng Gu	3rd Institue of Oceanography, CN
Nicole Trefault	Universidad Mayor, CL
Rut Akselman	INIDEP, AR
Rubén Lara	IADO, AR
Marcela Borel	INGEOSUR, AR


… and for your attention!