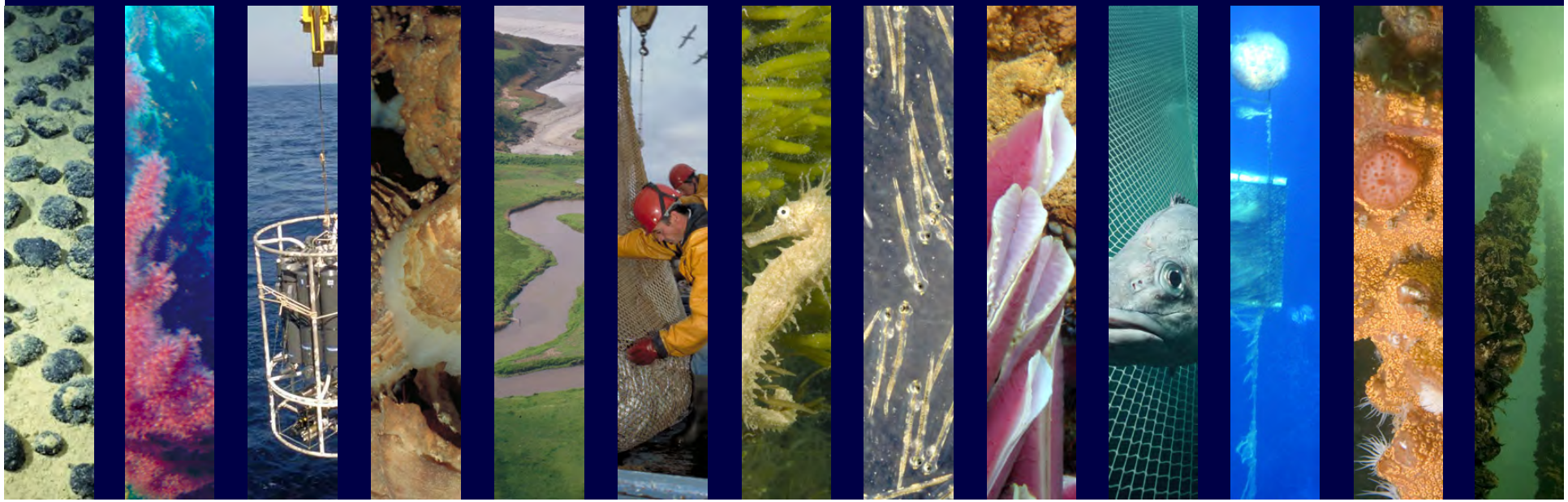
Contributions to the characterisation of risks posed by marine biotoxins

Philipp Hess

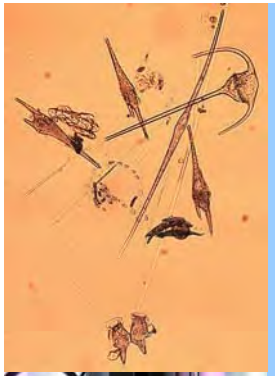
Presentation of the Habilitation to Direct Research

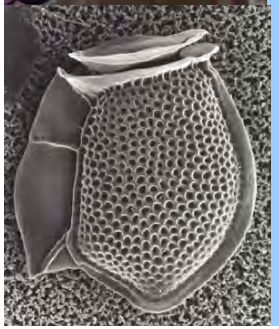
Ifremer, Atlantic Centre – University of Nantes



Harmful Algal Blooms

Efflorescences d’algues toxiques

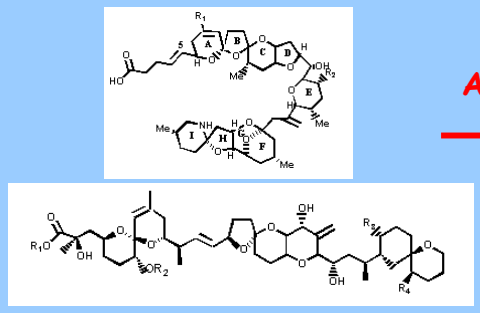




Phycotoxins – micro-algal toxins

Production

Phytoplanctonic cells

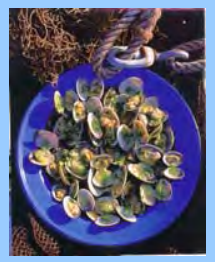


Marine biotoxins

Accumulation

Filter-feeding bivalves

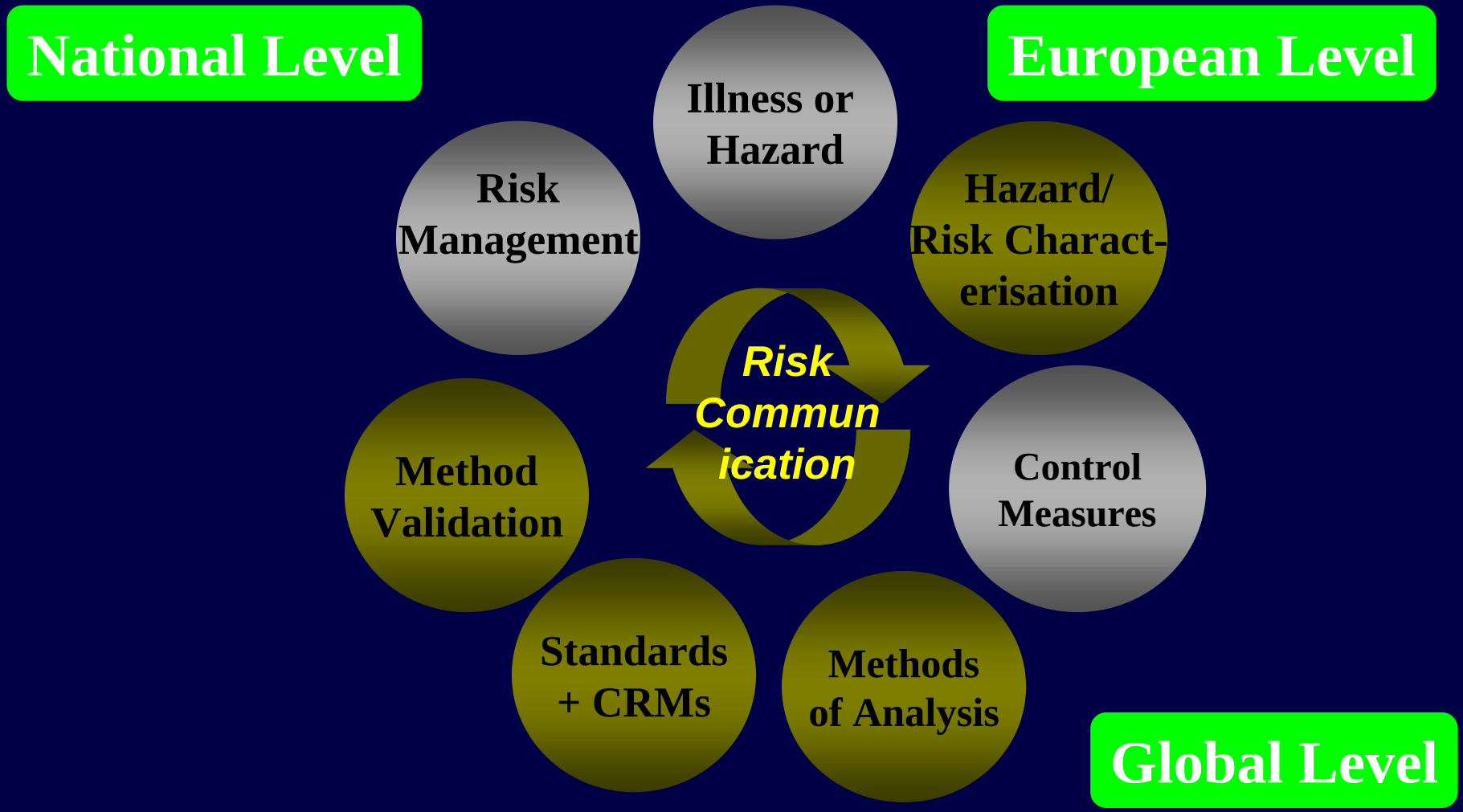
Processing, cooking etc.

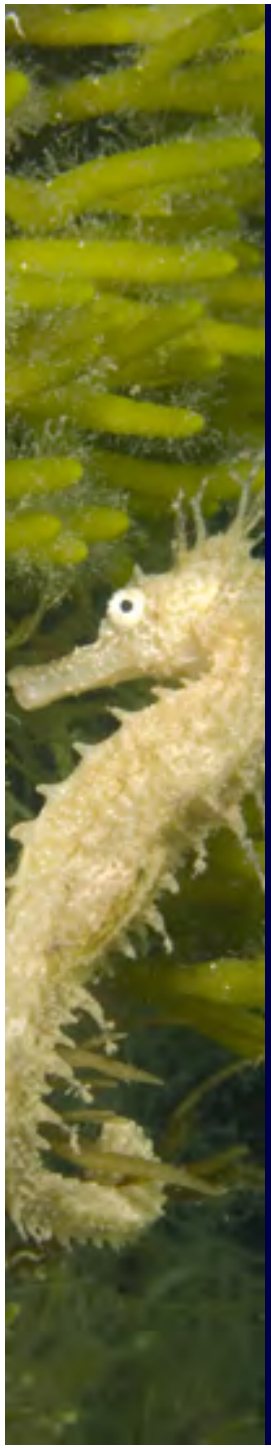


Food poisoning

Ideal Sequence of Official Food Control Measures

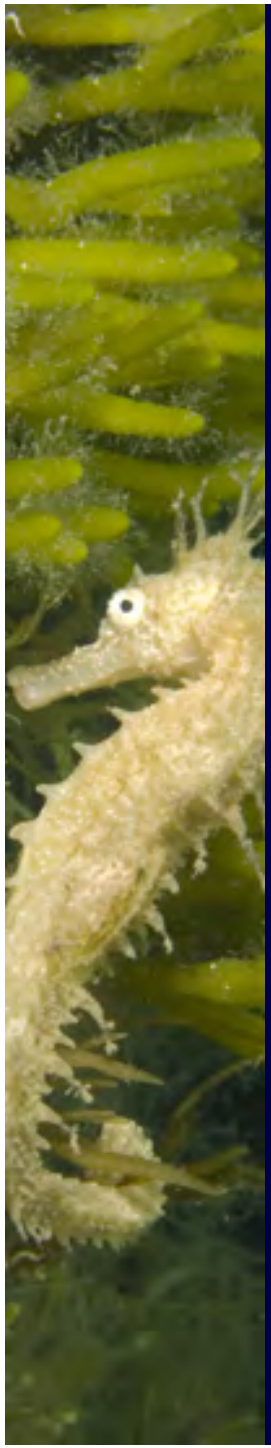
Ideal Sequence of Official Food Control Measures





Contents

- ✓ CV and scientific productivity
- ✓ Analysis of phycotoxins, natural products
- ✓ Quality Control (calibrants & reference materials)
- ✓ Comparison of detection techniques
- ✓ Characterisation of toxin hazards
- ✓ Outlook



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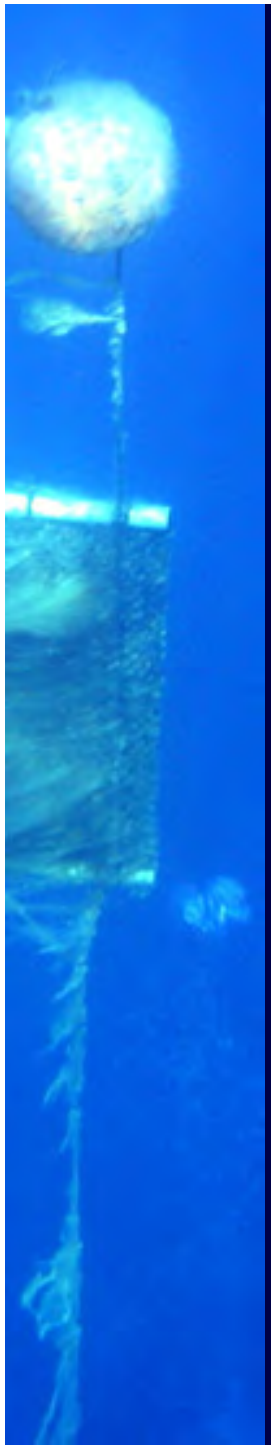
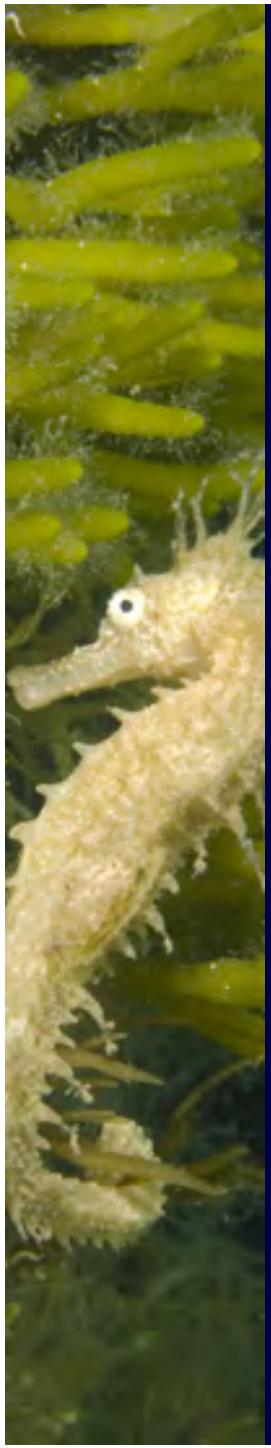


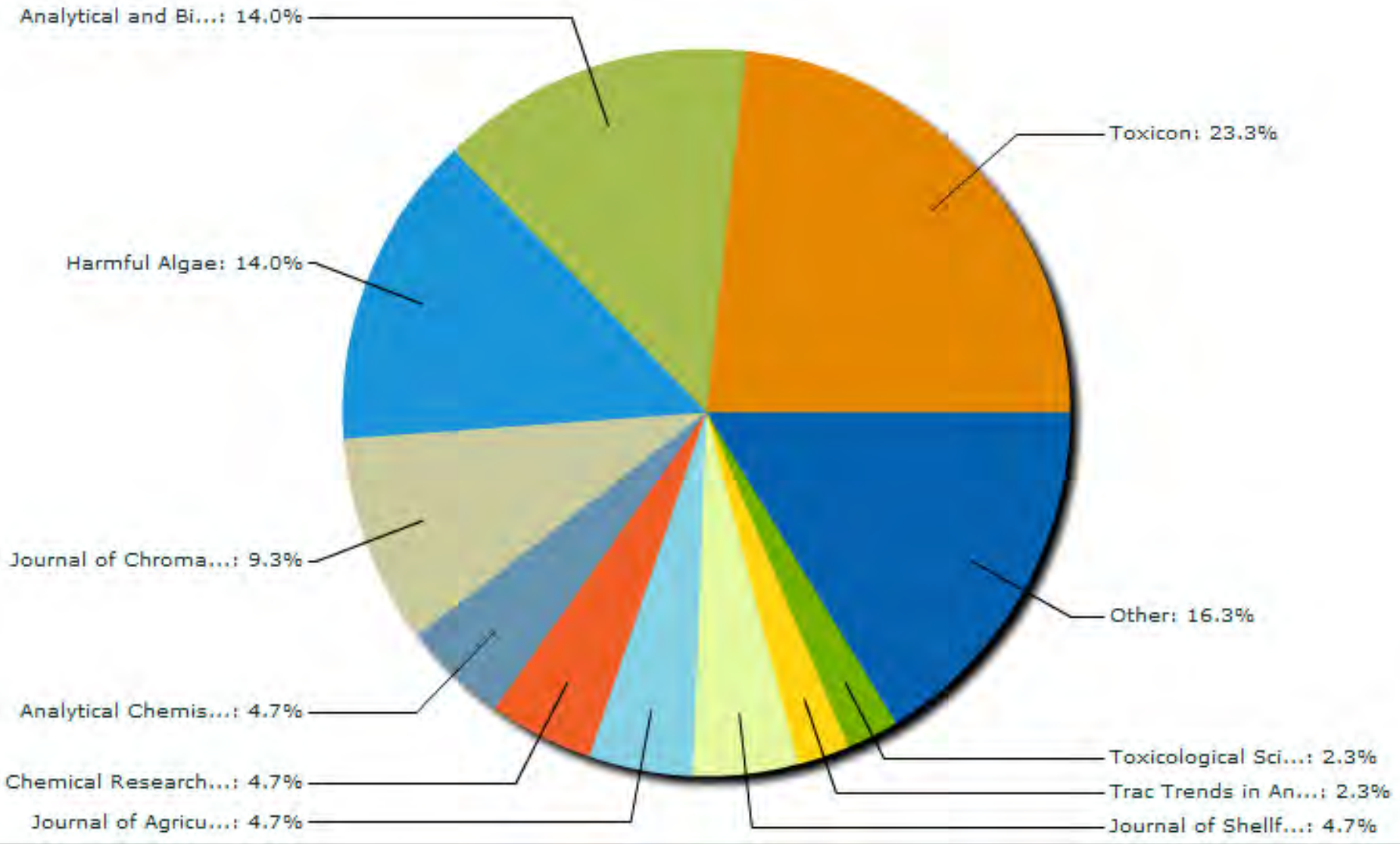
Univ. Saarland, DE (Vordiplom Chemie)	1987 – 1990	BSc general chemistry
EHICS, FR (Diplôme d'Ingénieur)	1990 - 1993	Chemical engineering Specialisation analytical chemistry
Robert Gordon Univ. Aberdeen, UK (PhD)	1993 - 1998	Chemical contaminants marine environment
Marine Laboratory phycotoxins	1998 - 2001	Team leader,
Marine Institue, IE phycotoxins	2001 - 2008	Team leader,
Ifremer, FR	2008 – 2010	Head of Dept. Environnement Microbiology & Phycotoxins

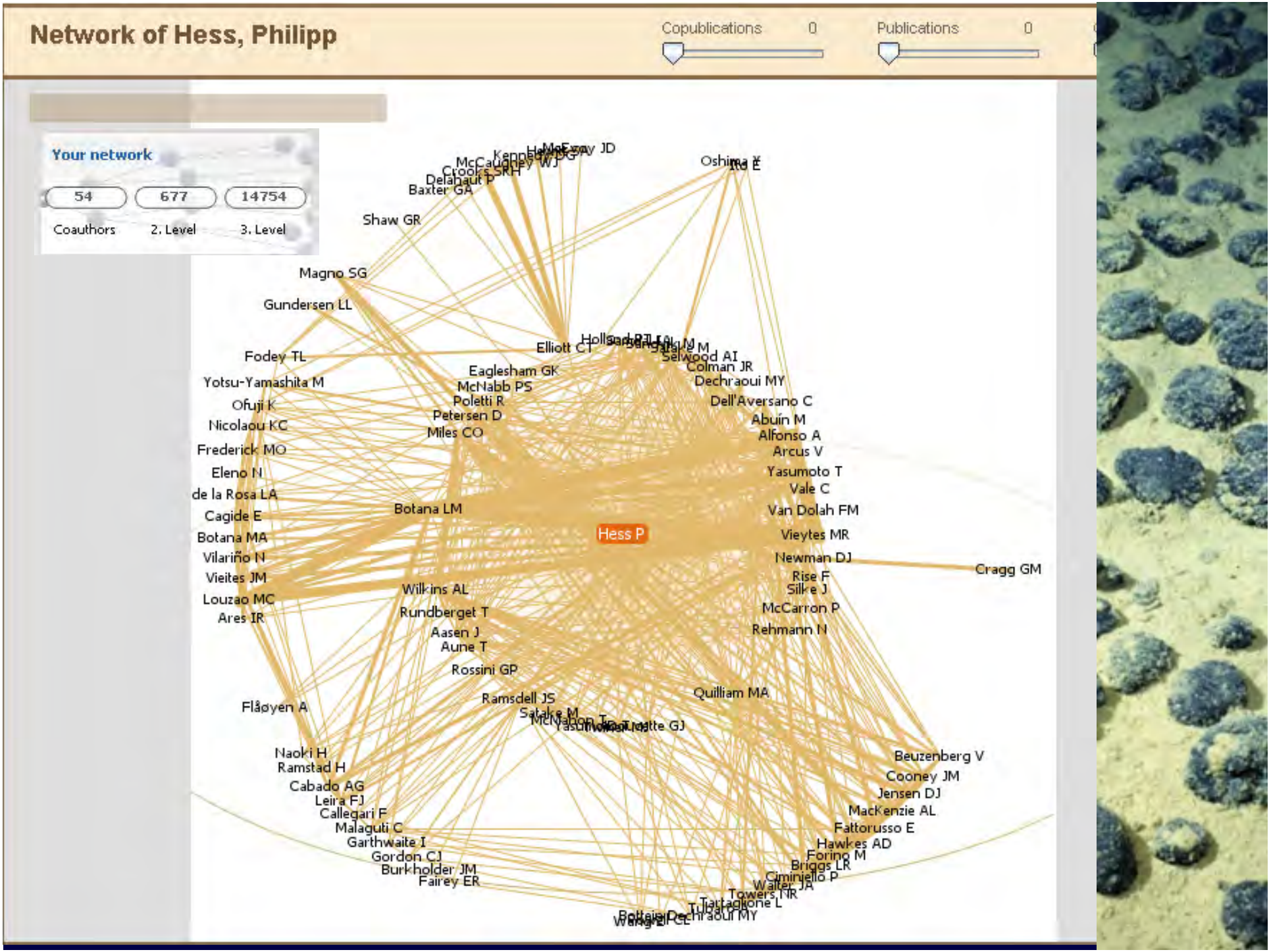


Academic achievements & activities

- ✓ Over 40 peer-reviewed publications, > 130 scientific contributions
- ✓ (Co-)supervision of 1 postdoc, 4 PhD, 2 MSc and 23 undergraduate students completed, 2 PhDs ongoing
- ✓ 9 multi-disciplinary projects managed in 10 years
- ✓ Co-organised 4 intl. conferences, 12 natl. workshops
- ✓ Expertise for many natl. & intl. food safety organisations (EFSA, ECVAM, FAO/IOC/WHO, AOAC, ANSES, QUASIMEME, MSSC, FSAI, UK-COT, FSA-UK, FSA-Scotland)
- ✓ Editorial board member of “Marine Drugs”

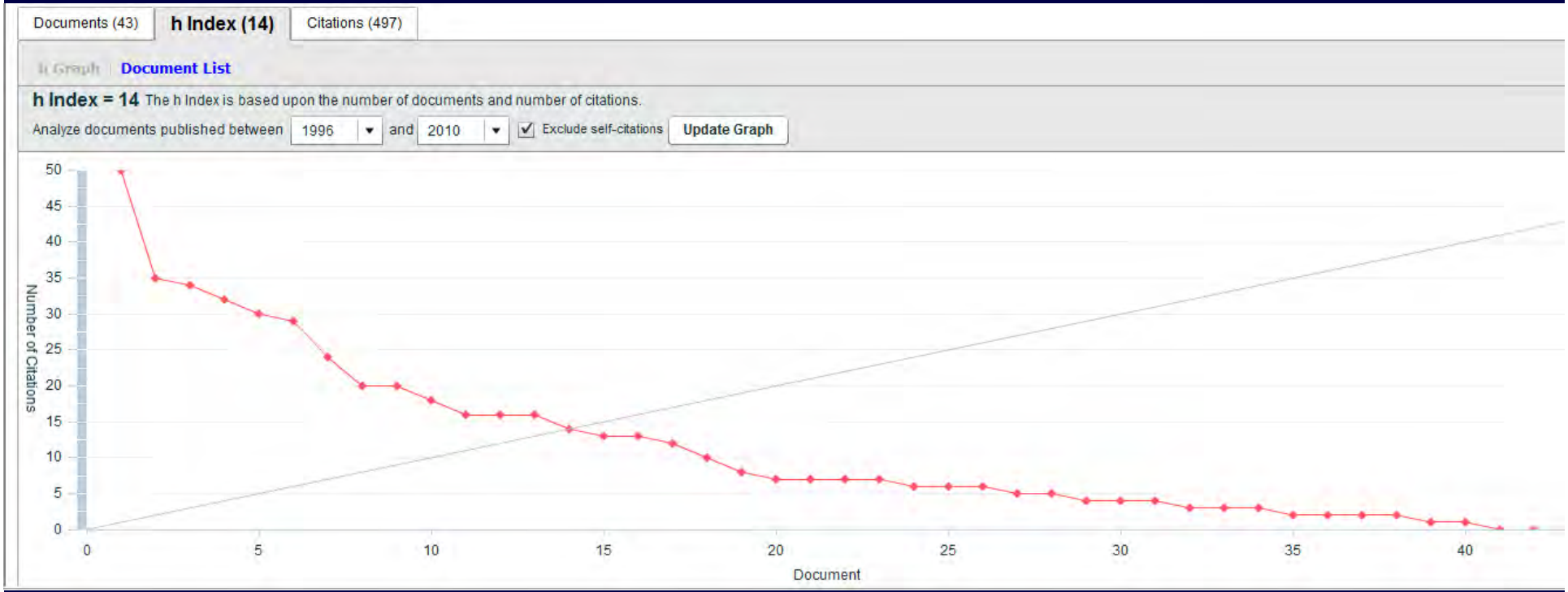
43 Publications – partitioned by journal published in




Network of Hess, Philipp
Copublications 0
Publications 0
Your network
54
677
14754
Coauthors
2. Level
3. Level
Shaw GR
Magno SG
Gundersen LL
Fodey TL
Yotsu-Yamashita M
Ofuji K
Nicolaou KC
Frederick MO
Eleno N
de la Rosa LA
Cagide E
Botana MA
Vilariño N
Vieites JM
Louzao MC
Ares IR
Botana LM
Wilkins AL
Rundberget T
Aasen J
Aune T
Rossini GP
Flåøyen A
Naoki H
Ramstad H
Cabado AG
Leira FJ
Callegari F
Malaguti C
Garthwaite I
Gordon CJ
Burkholder JM
Fairey ER
Eaglesham GK
McNabb PS
Poletti R
Petersen D
Miles CO
Hess P
Oshima Y
Ito E
Elliott CT
Selwood AI
Colman JR
Dechraoui MY
Dell'Aversano C
Abuin M
Alfonso A
Arcus V
Yasumoto T
Vale C
Van Dolah FM
Vieytes MR
Newman DJ
Rise F
Silke J
McCarron P
Rehmann N
Cragg GM
Quilliam MA
Ramsdell JS
Beuzenberg V
Cooney JM
Jensen DJ
MacKenzie AL
Fattorusso E
Hawkes AD
Forino M
Briggs LR
Ciminiello P
Walter JA
Towers NR
Tartaglione L

BioMed Experts geographical networking plot

Hirsch-index
a quantitative measure of the
<u>impact</u> of publications



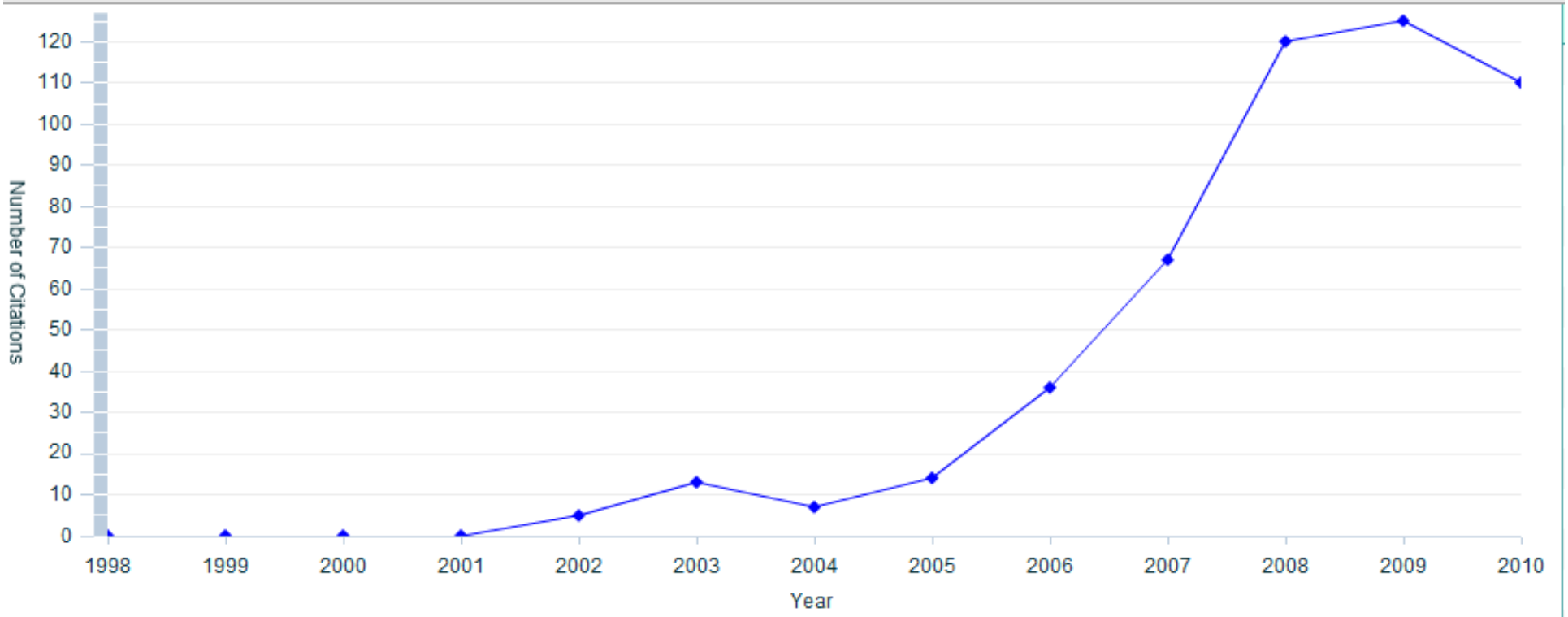
Source: http:/www.scopus .com

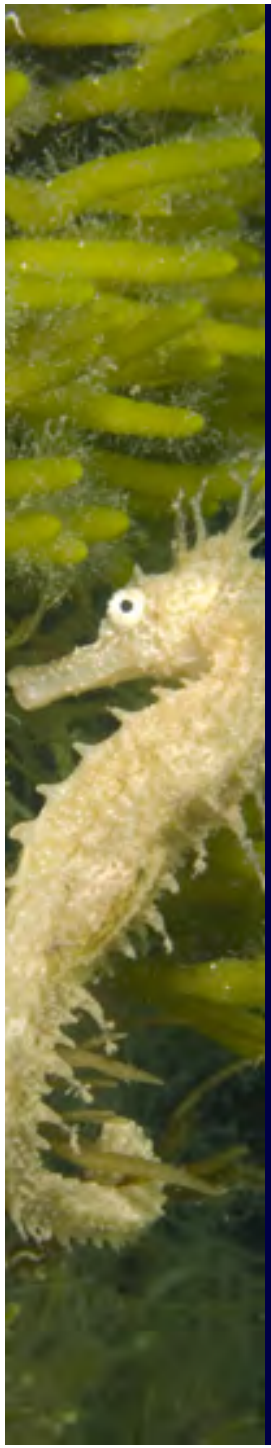
Evolution of citations

Documents (43) | h Index (14) | **Citations (497)**

Citations per year The Citations Graph shows the total number of citations received per year for an author's published works.

Analyze documents published between: 1996 and 2010 Update Graph





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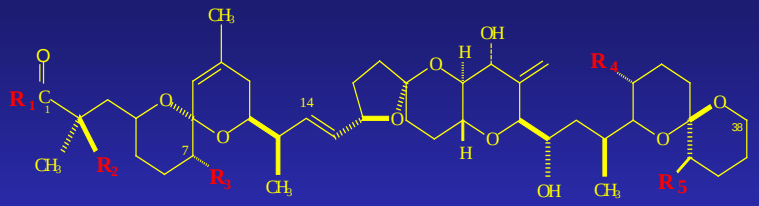
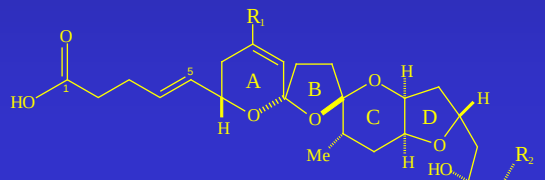
Chemical Structures of Phycotoxins

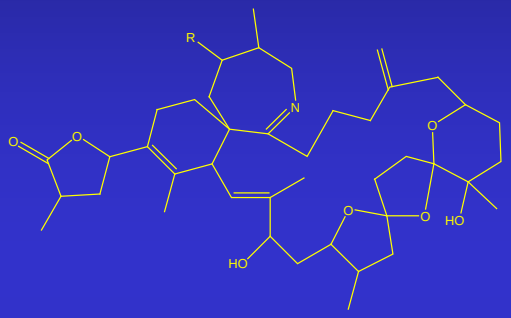
Lipophilic



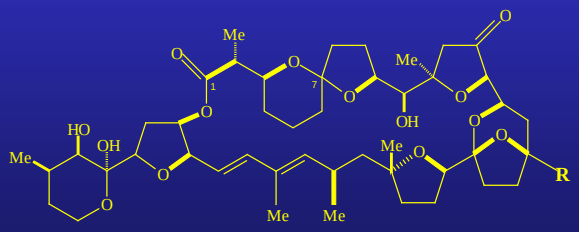
OA group (DSP)



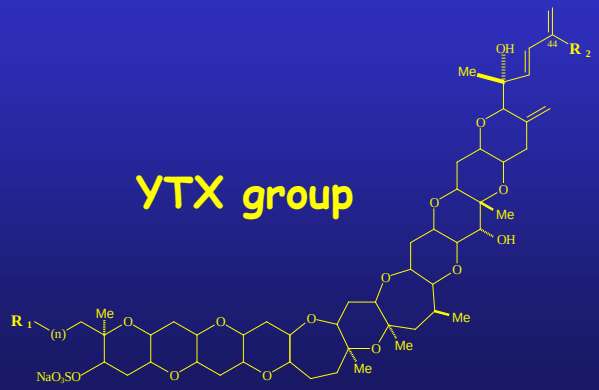
AZA group (AZP)



Cyclic imine group

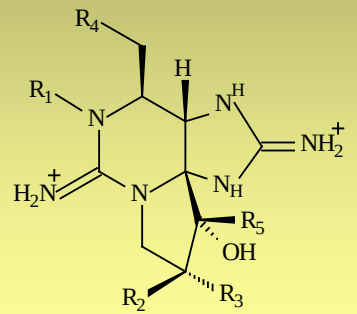


PTX group

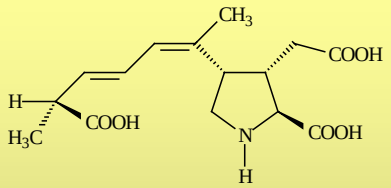


YTX group

Hydrophilic

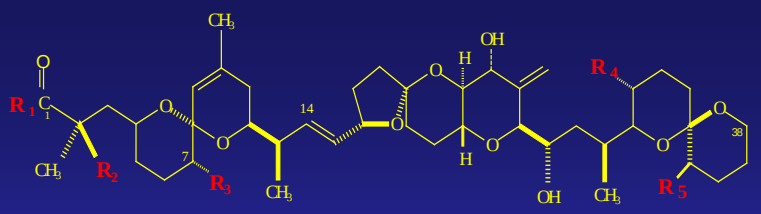


STX group (PSP)



DA group (ASP)

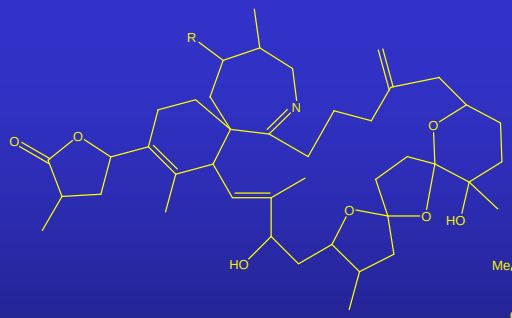
Acute Effects (within 30 min to 4 h)



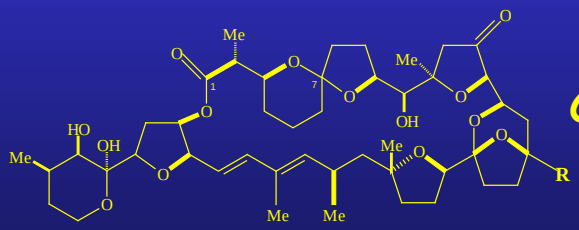
OA group (DSP)

AZA group (AZP)

Diarrhea
Nausea
vomiting
Stomach cramps

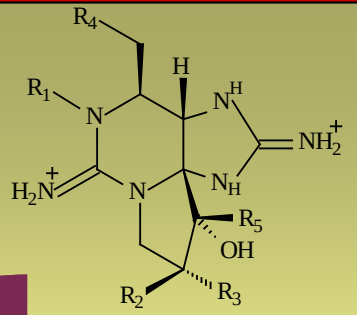


Cyclic imine group



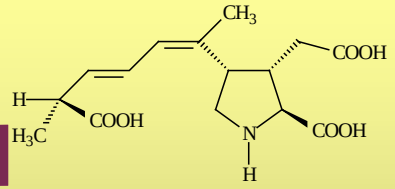
PTX group

YTX group



STX group (PSP)

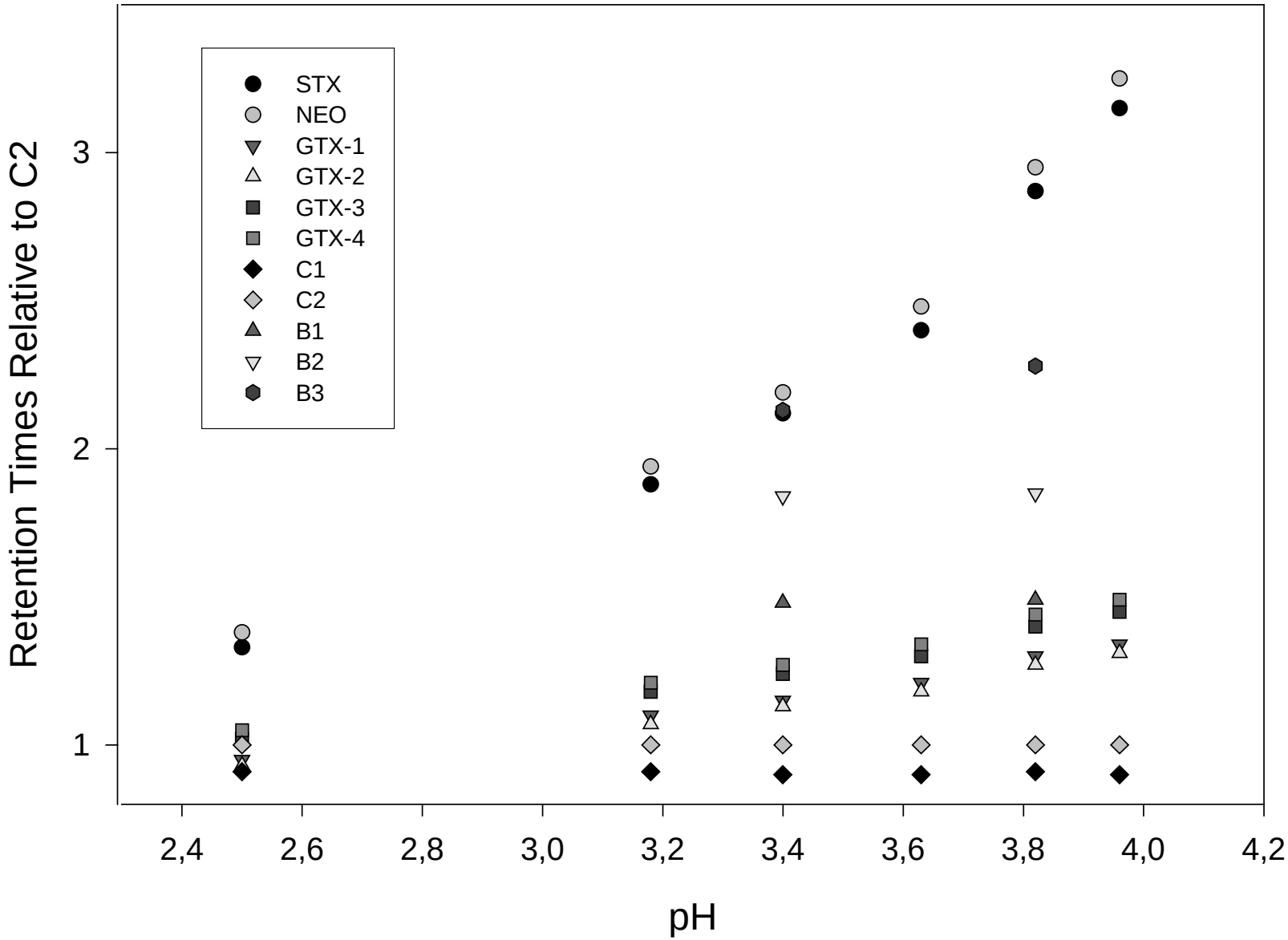
Sensory effects
Paralysis (death)



DA group (ASP)

Diarrhea, vomiting
Permant loss of
short-term memory

HILIC Chromatography of Saxitoxins



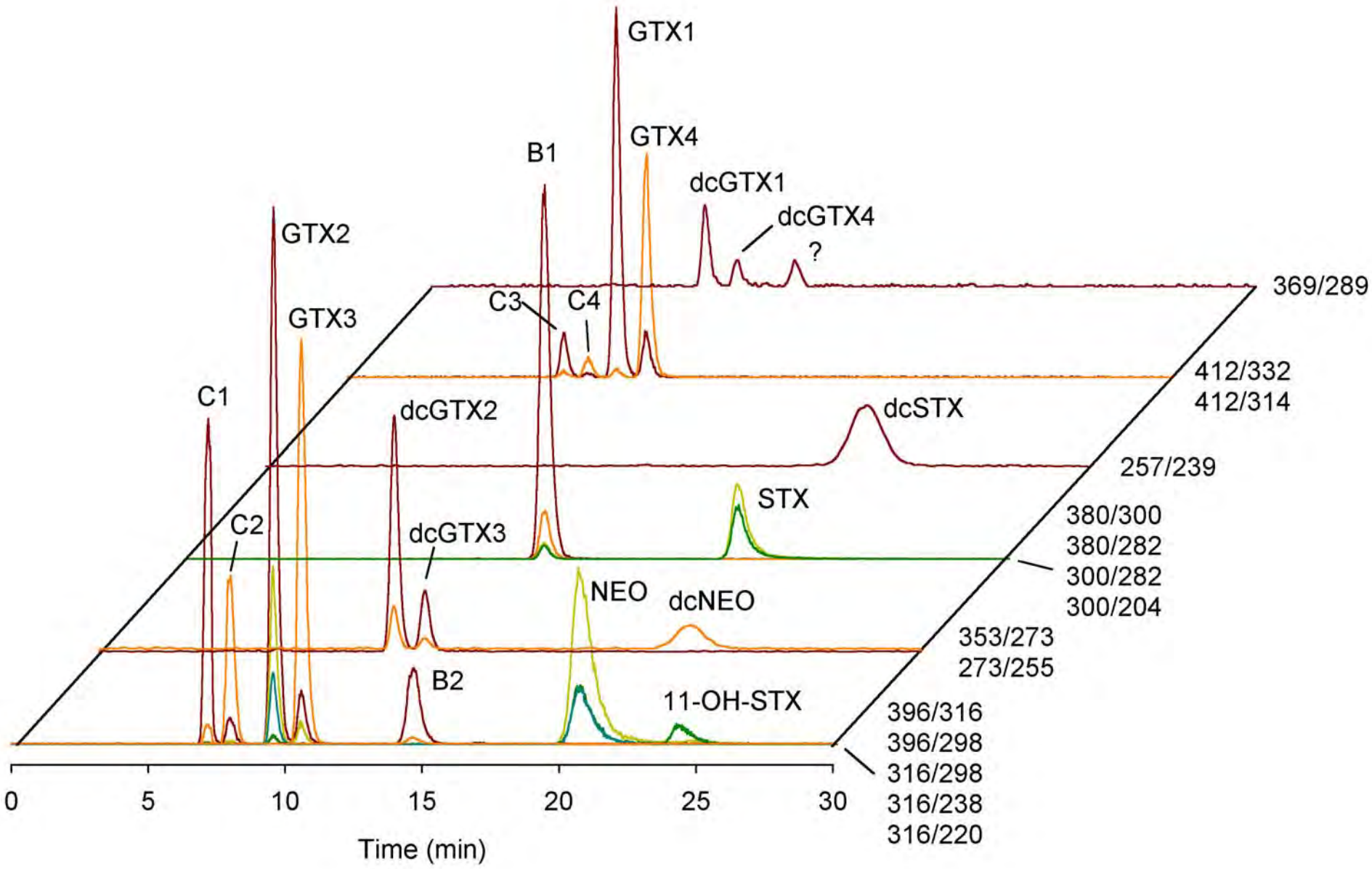

GTX1
GTX4
B1
dcGTX1
dcGTX4
?
GTX2
GTX3
C3
C4
C1
dcGTX2
dcSTX
C2
dcGTX3
STX
NEO
dcNEO
B2
11-OH-STX
369/289
412/332
412/314
257/239
380/300
380/282
300/282
300/204
353/273
273/255
396/316
396/298
316/298
316/238
316/220
0
5
10
15
20
25
30
Time (min)

Table 2.1.2. Estimated detection limits (LOD, S/N = 3) for saxitoxin group toxins (nanomolar concentration, 5 µL injected on column) for tandem MS (Dell'Aversano et al., 2005) and LC with post-column oxidation and fluorescence detection (Oshima, 1995).

	MS-SRM	LC-FLD
Toxin	API4000	Post-column
STX	20	60
NEO	30	60
GTX2	20	20
GTX3	10	5
GTX1	10	20
GTX4	5	30
B1	10	100
C1	20	30
C2	10	20

Influence of matrix on ion suppression in LC-MS analysis of azaspiracids - BIOTOX (EU FP7)

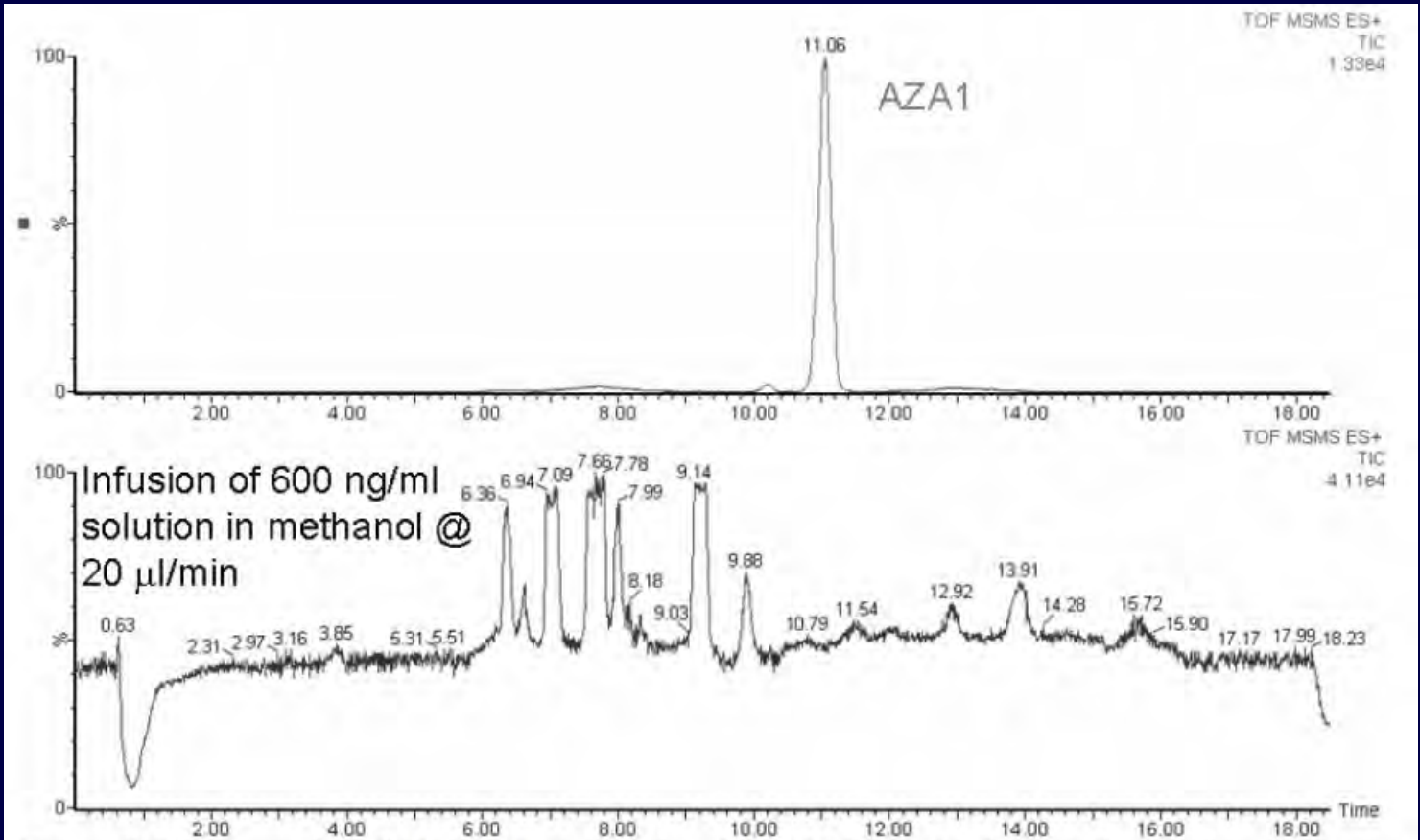


Figure 2.2.8. Examples of (A) AZA1 standard and (B) AZA1 post column infusion chromatograms obtained by step gradient HPLC with Q-Tof detection.

Fux E., Rode D., Bire R., Hess P. (2008) Food Addit. Contam. 25 (8), 1024-1032.

→Matrix effect not necessarily easy to measure by any technique

Influence of sample strength on matrix effect in LC-MS

BIOTOX (EU FP7)

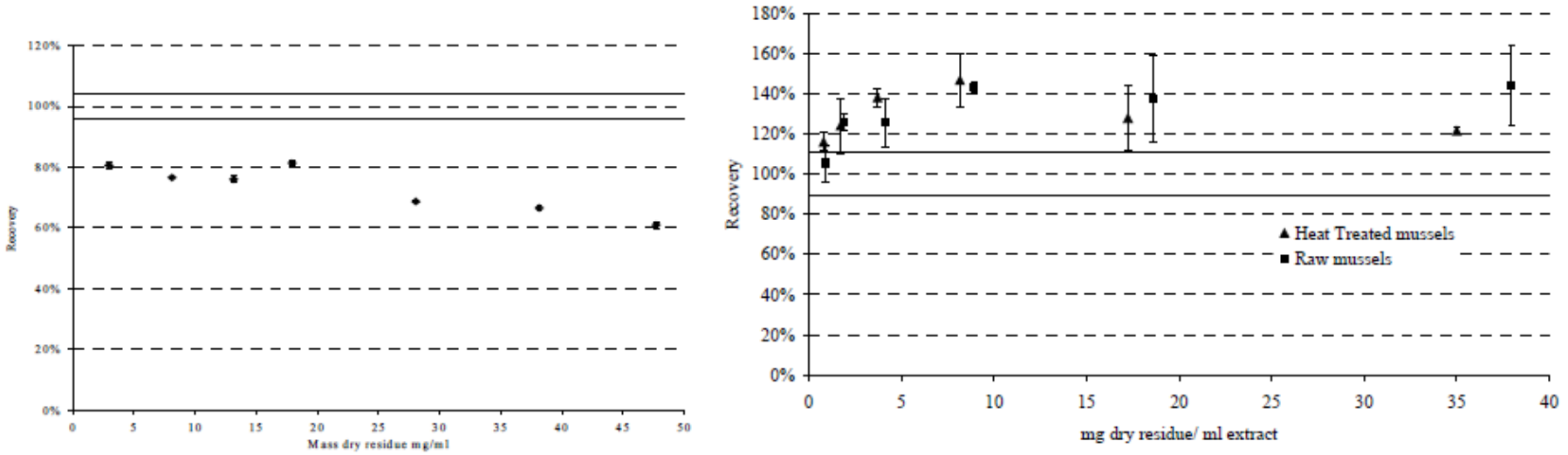


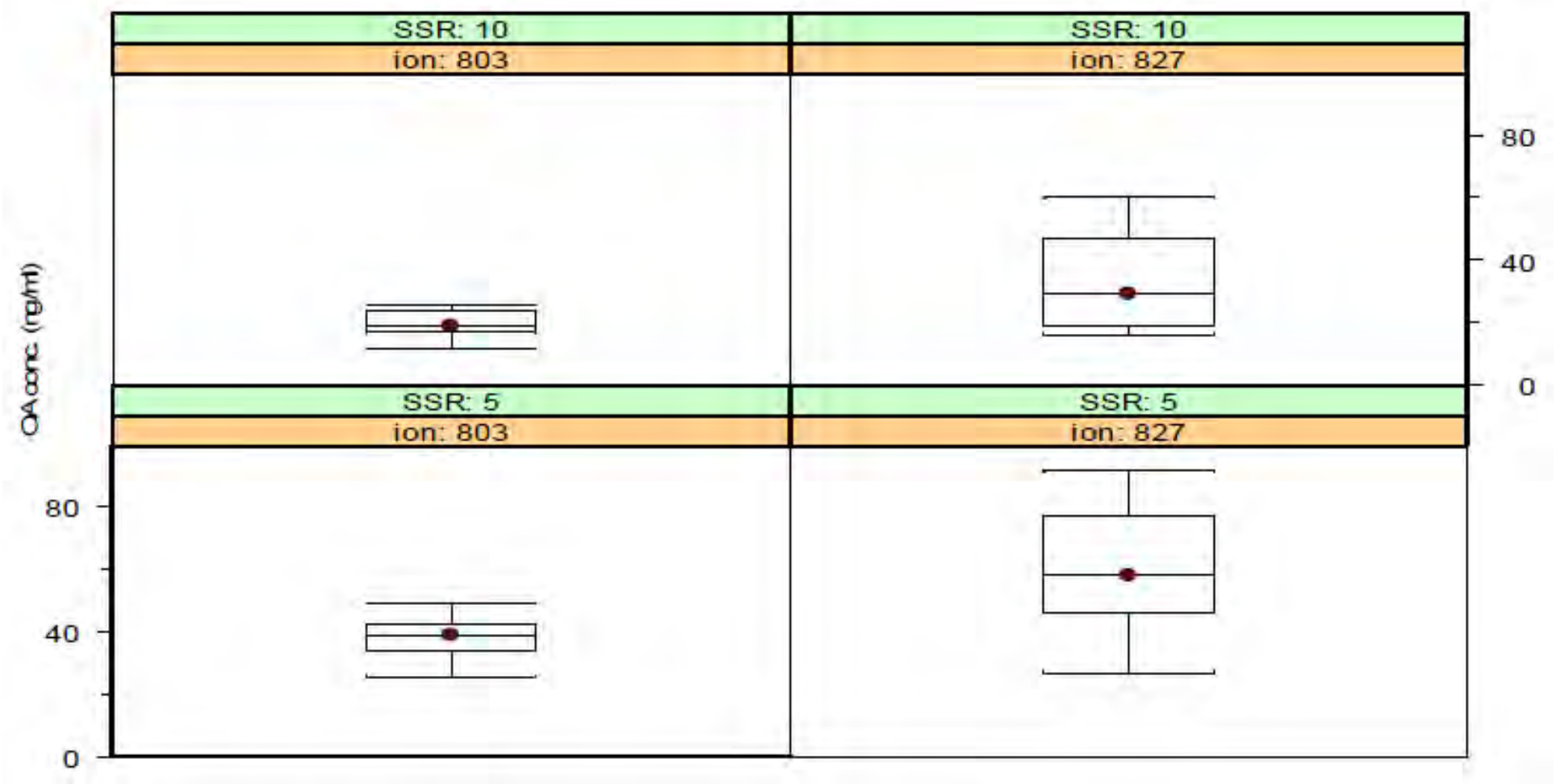
Figure 2.2.9. Post extraction addition of AZA (left graph) and OA (right graph) analysed using step gradient HPLC conditions and Q-Tof detection. Bold lines represent the precision obtained

Fux E., Rode D., Bire R., Hess P. (2008) Food Addit. Contam. 25 (8), 1024-1032.

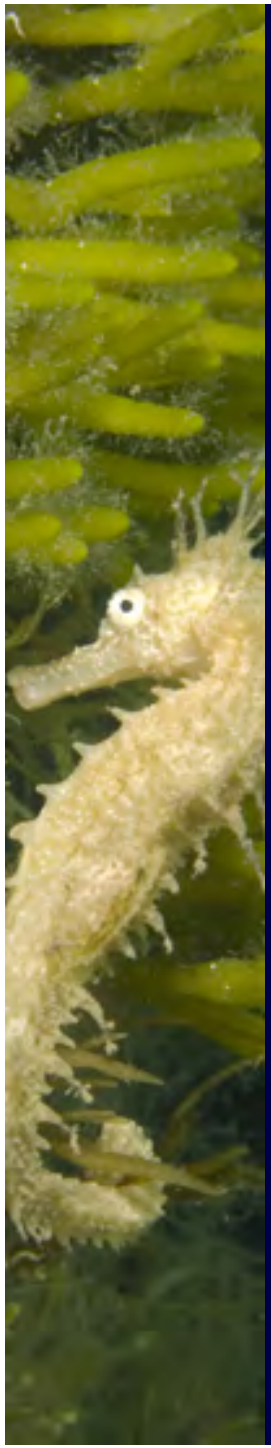
→Large dilution necessary to overcome matrix effects in crude extracts (< 1 mg sample / mL extract) !!

Influence of ionisation mode on between laboratory variability

BIOTOX (EU FP7) – 17 Participants, round 1



→ *Negative ionisation mode necessary for Okadaic Acid*



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Purification of Azaspiracids –preparative scale

Partitioning 2
(80% aq. MeOH
- hexane)

Partitioning 1
(water –
ethylacetate)

Extraction
(Large-scale)

Purification of Azaspiracids

Reverse-phase HPLC
(ODP, Showa-denko)

Cation Chromatography
(Toyopearl CM650)

Anion Chromatography
(Toyopearl DEAE)

Rev Chromatography
(Develosil Lop ODS)

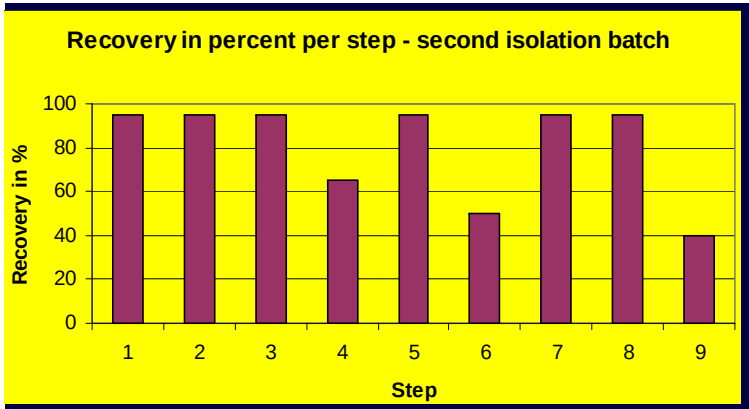
Size- Chromatography
(HW40-SF)

Nor chromatography
(Silica Gel)

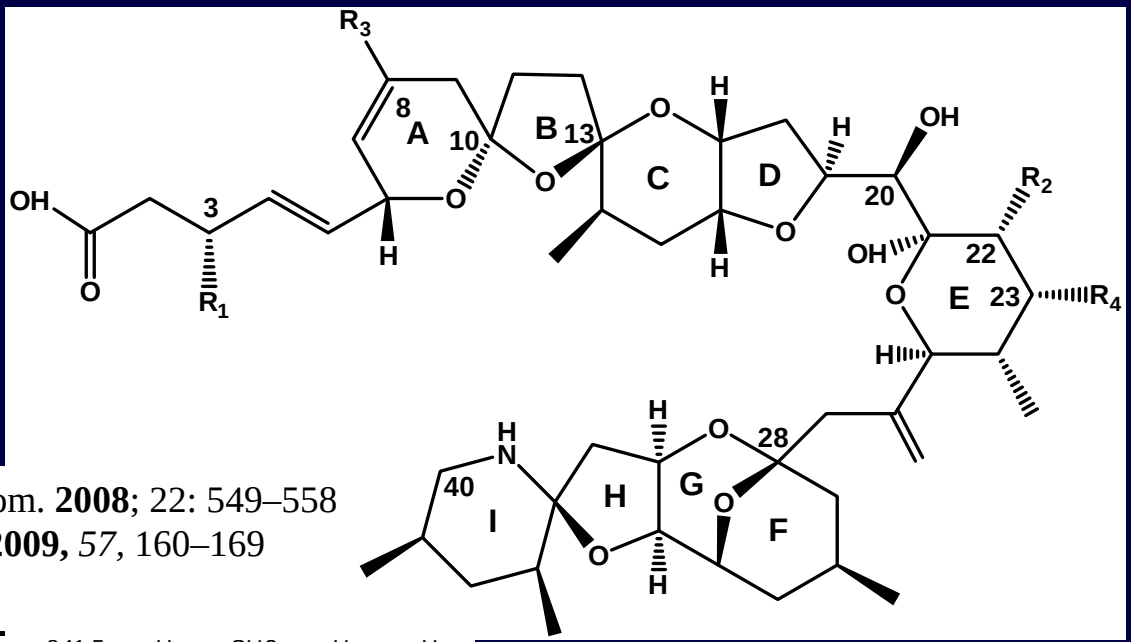
Par (80% aq. MeOH - hexane)

Par (ethylacetate)

E (Large-scale)



9 % overall recovery 400 µg from 4.5 mg

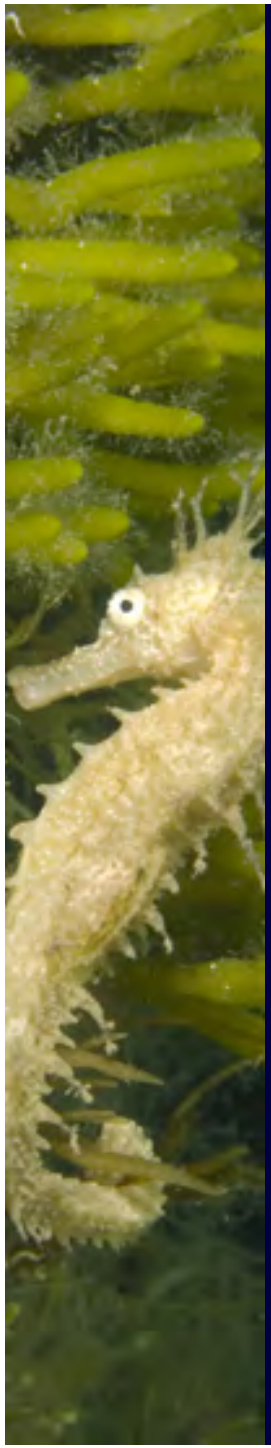


Rehmann et al., Rapid Commun. Mass Spectrom. **2008**; 22: 549–558
McCarron *et al.*, 2009, *J. Agric. Food Chem.* **2009,** *57,* 160–169

Abbreviation	Chemical name					
AZA1	Azaspiracid	841.5	H	CH3	H	H
AZA2	8-methyl-azaspiracid	855.5	CH3	CH3	H	H
AZA3	22-desmethyl-azaspiracid	827.5	H	H	H	H
AZA4	22-desmethyl-3-hydroxy-azaspiracid	843.5	H	H	OH	H
AZA5	*22-desmethyl-23-hydroxy-azaspiracid*	*843.5*	*H*	*H*	*H*	*OH*
AZA6	22-desmethyl-8-methyl-azaspiracid	841.5	CH3	H	H	H
AZA7	*3-hydroxy-azaspiracid*	*857.5*	*H*	*CH3*	*OH*	*H*
AZA8	*23-hydroxy-azaspiracid*	*857.5*	*H*	*CH3*	*H*	*OH*
AZA9	22-desmethyl-3-hydroxy-8-methyl-azaspiracid	857.5	CH3	H	OH	H
AZA10	*22-desmethyl-23-hydroxy-8-methyl-azaspiracid*	*857.5*	*CH3*	*H*	*H*	*OH*
AZA11	*3-hydroxy-8-methyl-azaspiracid*	*871.5*	*CH3*	*CH3*	*OH*	*H*
AZA12	*23-hydroxy-8-methyl-azaspiracid*	*871.5*	*CH3*	*CH3*	*H*	*OH*
AZA13	*22-desmethyl-3,23-dihydroxy-azaspiracid*	*859.5*	*H*	*H*	*OH*	*OH*
AZA14	*3,23-dihydroxy-azaspiracid*	*873.5*	*H*	*CH3*	*OH*	*OH*
AZA15	*22-desmethyl-3,23-dihydroxy-8-methyl-azaspiracid*	*873.5*	*CH3*	*H*	*OH*	*OH*
AZA16	*3,23-dihydroxy-8-methyl-azaspiracid*	*877.5*	*CH3*	*CH3*	*OH*	*OH*
AZA17**	carboxy-22-desmethyl-azaspiracid	871.5	H	COOH	H	H
*AZA18**	*carboxy-azaspiracid*	*885.5*	*H*	*CH3*	*H*	*H*
AZA19**	carboxy-22-desmethyl-8-methyl-azaspiracid	885.5	CH3	COOH	H	H
*AZA20**	*carboxy-8-methyl-azaspiracid*	*899.5*	*CH3*	*CH3*	*H*	*H*
AZA21**	carboxy-22-desmethyl-3-hydroxy-azaspiracid	887.5	H	COOH	OH	H
*AZA22**	*carboxy-3-hydroxy-azaspiracid*	*901.5*	*H*	*CH3*	*OH*	*H*
AZA23**	carboxy-22-desmethyl-3-hydroxy-8-methyl-azaspiracid	901.5	CH3	COOH	OH	H
*AZA24**	*carboxy-3-hydroxy-8-methyl-azaspiracid*	*915.5*	*CH3*	*CH3*	*OH*	*H*

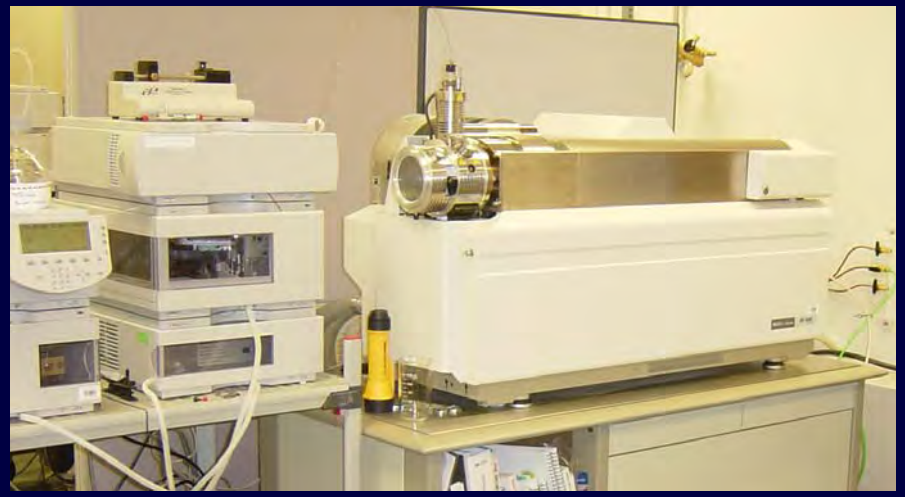
** these analogs have not been detected in samples*

*** these tentative structures are based on in-direct evidence from D/H switching experiments as part of these studies*



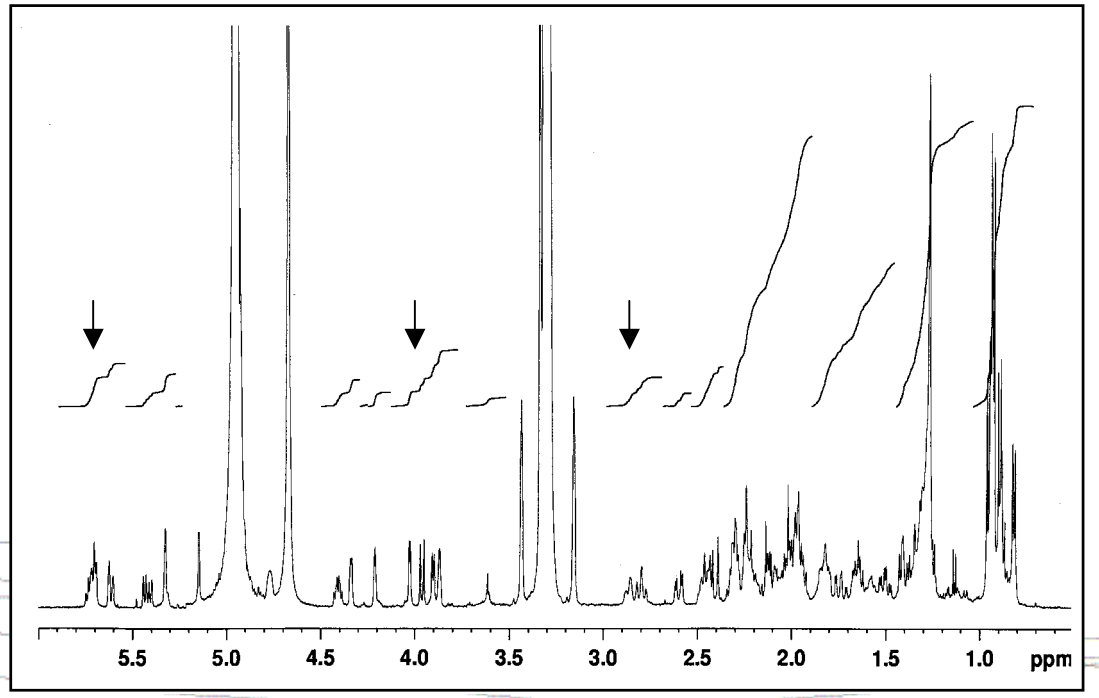
Certification

- **Assessment of purity**
  - **NMR**
  - **LC-MS**



Primary Calibrants: Quantitative NMR

I.W. Burton, M.A. Quilliam and J.A. Walter
***Analyt. Chem.* 77**, 3123-3131 (2005).



National Research Council Canada
Conseil national de recherches Canada

Certification – LC-MS/MS

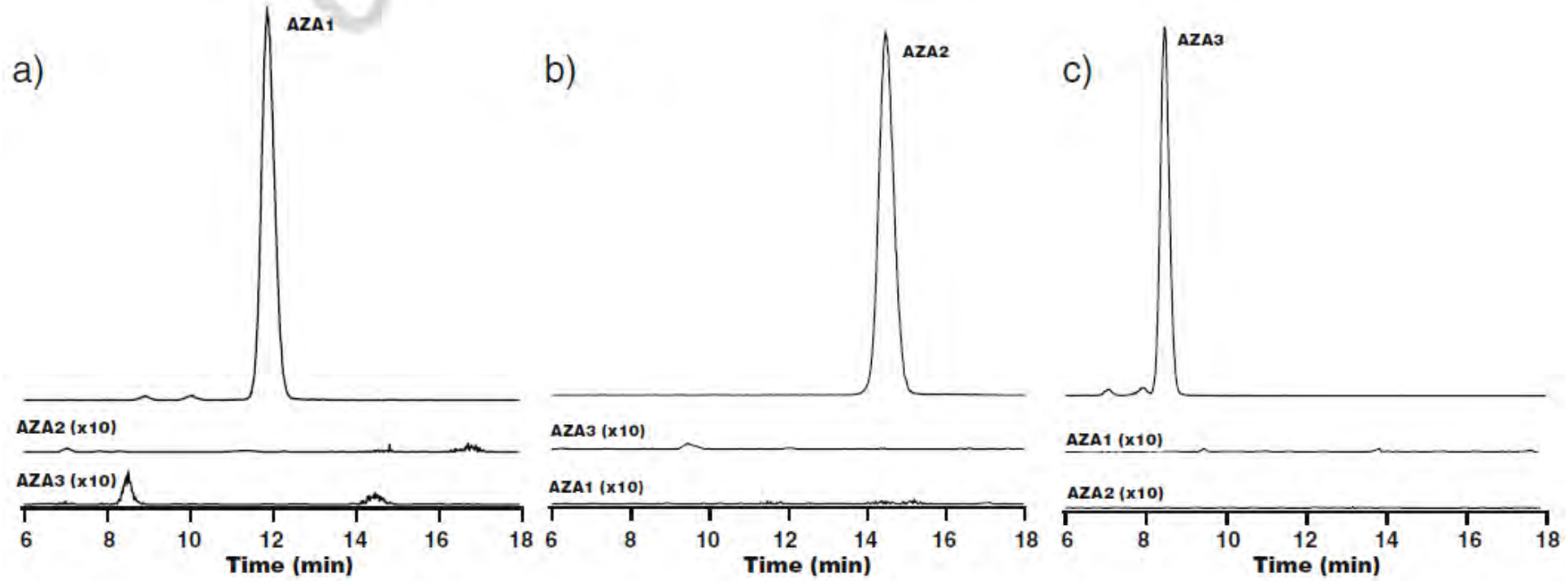
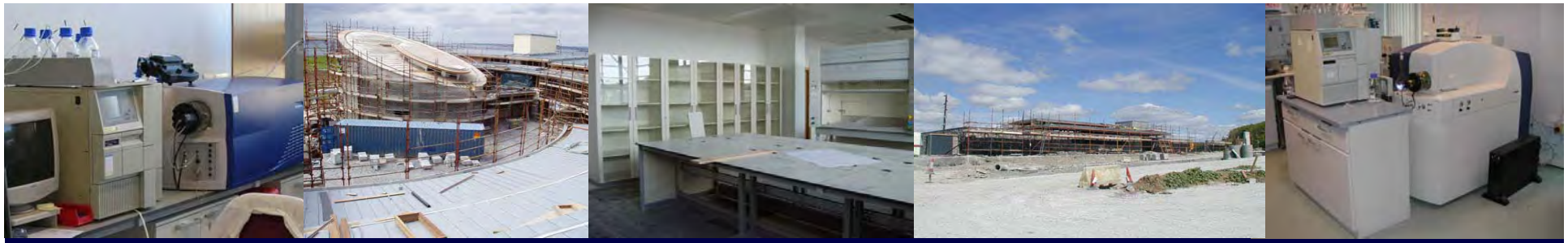


Fig. 4 LC-MS analysis of NRC CRM-AZA1 (**a**), -AZA2 (**b**) and -AZA3 (**c**) using MRM at *m/z* 842.5>672.5, 856.5>672.5 and 828.5>658.5, respectively. The three MRM transitions were monitored in all three CRMs to assess potential contamination from other AZAs in the CRMs

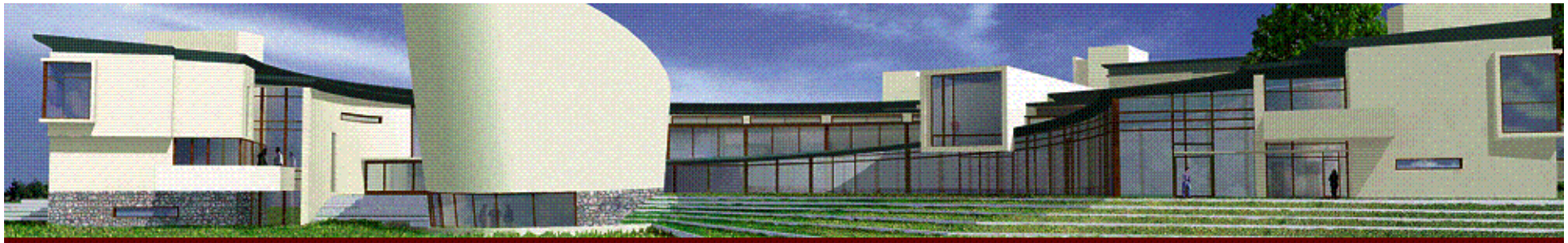
Perez R., Rehmann N., Crain S., LeBlanc P., Craft C., MacKinnon S., Reeves K., Burton I.W., Walter J.A., Hess P., Quilliam M.A. and Melanson J.E. (2010) The preparation of certified calibration solutions for azaspiracid 1, -2 and -3, potent marine biotoxins found in shellfish. Accepted for publication in Analytical and Bioanalytical Chemistry on 22-08-2010.



Operational Estimate of AZA1-use by LC-MS

If 50 laboratories use 10 μg per year

- ↳ 500 μg per year total consumption
- ↳ 2.5 mg / 500 μg = 5 years
- ↳ Current AZA-1 is used up < 5 years



QC-Tools: Tissue Reference Materials

A. Laboratory reference materials (LRMs)

- **Method validation (precision)**
- **Routine QC**

B. Interlaboratory RMs (ILRMs)

- **Intercomparison exercises**
- **Proficiency testing**

C. Certified reference material (CRMs)

- **Method validation (accuracy)**
- **Calibration**

Optimised procedure for wet in-house LRMs

Production of a RM for Shellfish Toxins

LRM Design
(shellfish species, bulk amount and portion size, toxin profile, concentrations, anticipated duration of use)

Gentle Heat Treatment
(e.g. steaming for 10 min., facilitates opening of shells and prevents enzymatic degradations)

Initial Blending
(e.g. household or Waring blender, mincing of streaky shellfish tissues)

Adjustment of water content to ca. 85 %
Addition of antioxidant and antibiotics

Fluidising
(UltraTurrax or Polytron
remove heavy particles, sand and broken shells)

Dispensing
(pre-labelled containers, peristaltic pump speeds up process, sample must be mixed/stirred during process)

Purging + Sealing of Containers
(argon or nitrogen purge, aluminium lids heat-sealed onto polypropylene vials, MK-1)

Frozen Storage
(e.g. box in chest freezer, -20 °C)

Characterisation
(homogeneity and stability testing)

SOP#: BCT-58

Hess P., McCarron P., Quilliam M.A. (2007) Fit-for-purpose Shellfish Reference Materials for Phycotoxins in Internal and External Quality Control. ***Anal. Bioanal. Chem.* 387, 2463-2474.**

Procedure for freeze-dried RMs @

Freeze drying

KFT (2.5-4%)

Crushing

Ball milling

PSA

Sieving (<125µm)

Turbula mixing

Filling and Sealing

Process control:
- PSA
- KFT

PSA = Particle Size Analysis; KFT = Karl Fischer Titration

Homogeneity testing of DA materials

Material	Average DA conc µg/g	SD	%CV
Wet	10.4	0.3	2.0
Freeze dried	56.5	1.6	2.8

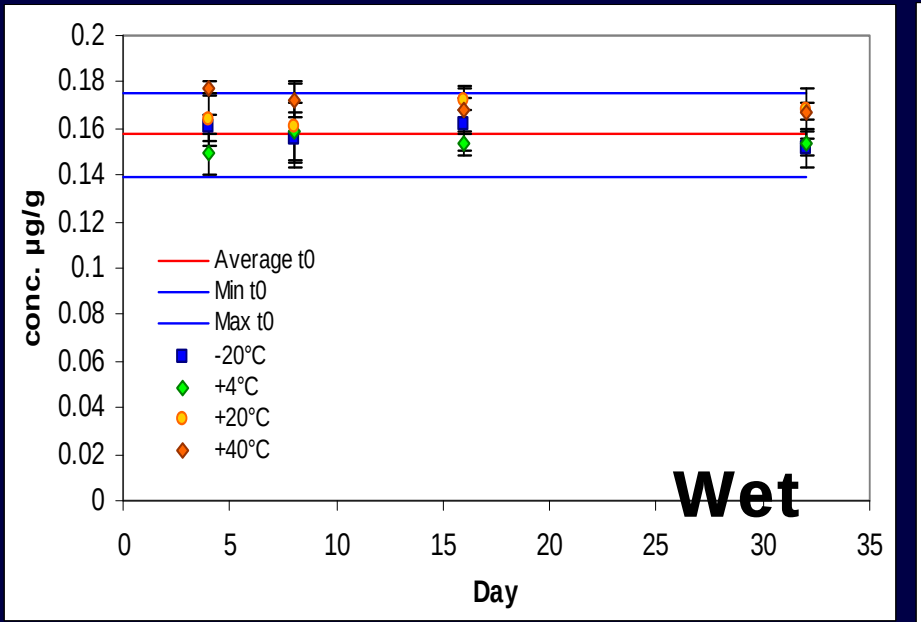
Homogeneity testing of OA / AZA materials (n=10)

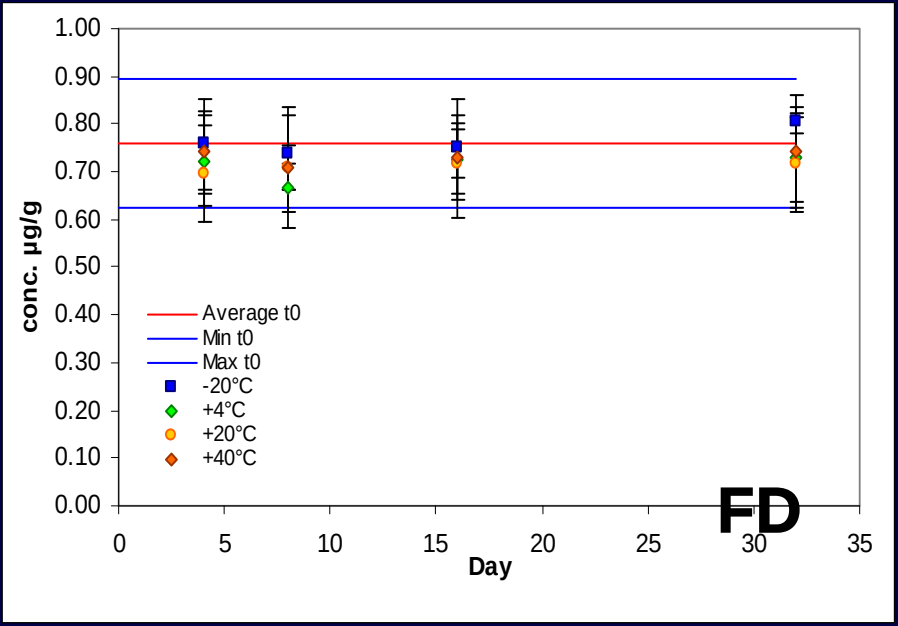
		AZA-3 [µg/g]	AZA-1 [µg/g]	AZA-2 [µg/g]	**AZP equiv. (µg/g)**	OA [µg/g]	DTX-2 [µg/g]	**OA equiv. (µg/g)**
Freeze Dried	Average	0.10	0.86	0.35	**1.64**	0.96	2.16	**3.12**
	Stdev	0.01	0.03	0.02	**0.05**	0.08	0.11	**0.15**
	%CV	*5.7*	*3.5*	*4.8*	***2.9***	*7.9*	*5.0*	***4.9***
Wet	Average	0.02	0.18	0.07	**0.34**	0.17	0.38	**0.55**
	Stdev	0.00	0.01	0.00	**0.02**	0.01	0.04	**0.04**
	%CV	*12.4*	*4.9*	*4.2*	***4.5***	*4.4*	*9.3*	***7.6***

- **Good homogeneity**
- **Higher RSD due to LC-MS method used**

Azaspiracid 1 in wet and freeze dried

AZA short termstability studies

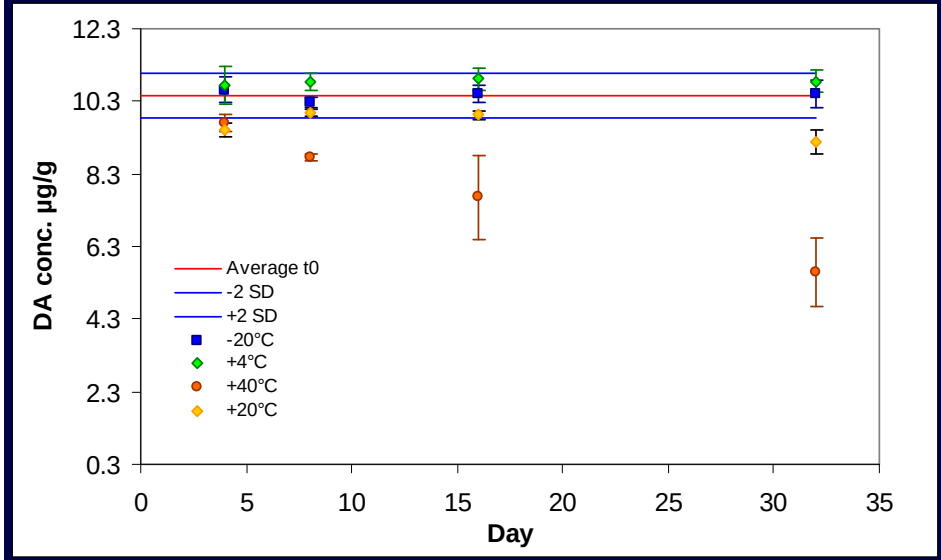


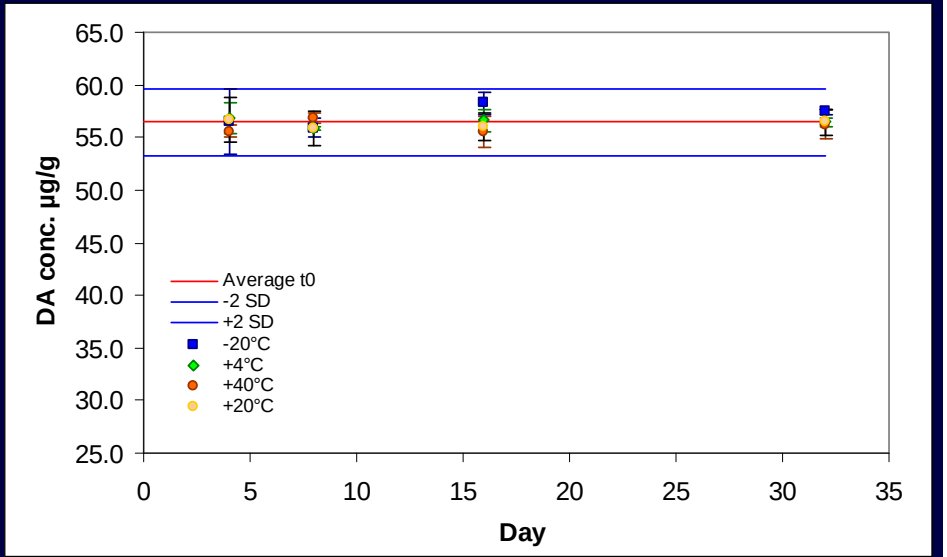


- **AZA-1 stable in both materials over short term**
- **AZA-2 stable over short term (data not shown)**
- **AZA-3 unstable in both materials (data not shown)**

DA short term stability studies

Wet vs freeze-dried

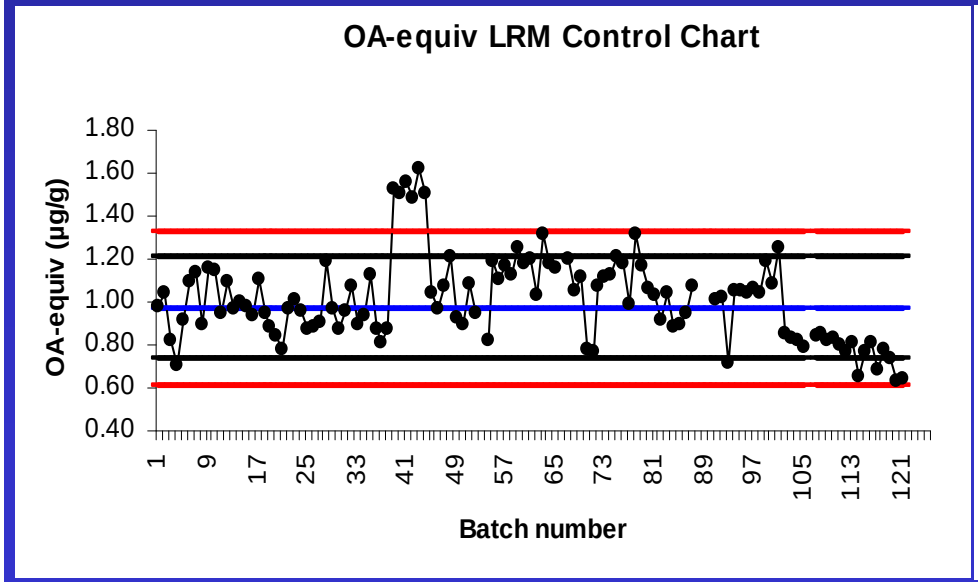


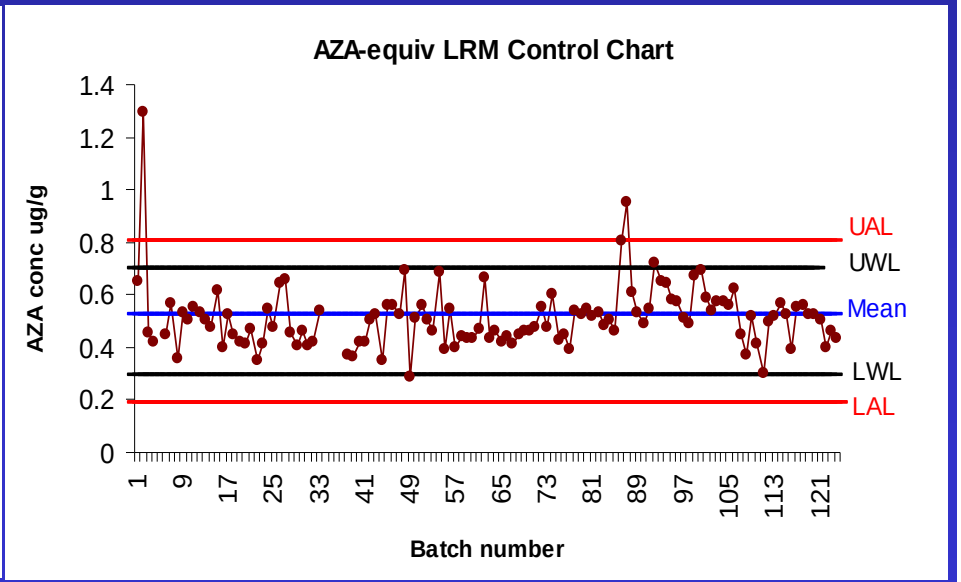


- No degradation of DA detected in wet RM at –20°C or +4°C
- Degradation of DA at higher temperatures in wet RM
- No degradation of DA at any temperature in freeze-dried RM

Overview of materials prepared

- A total of 18 OA/AZA RMs prepared
- Materials have been used for a variety of applications:
  - Single Laboratory Validation
  - Proficiency Testing
  - Interlab/Collaborative studies (12 BIOTC
  - Feasibility of CRM production



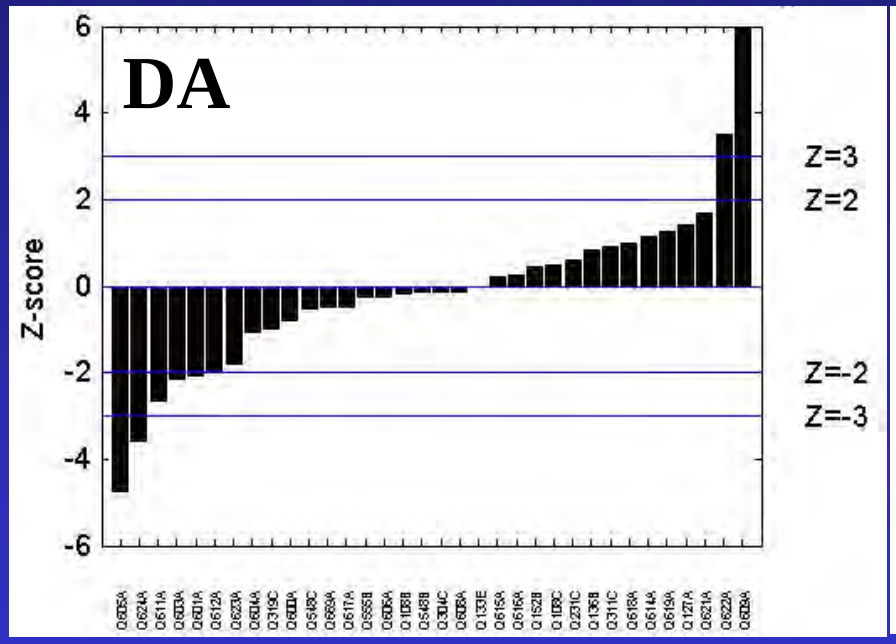


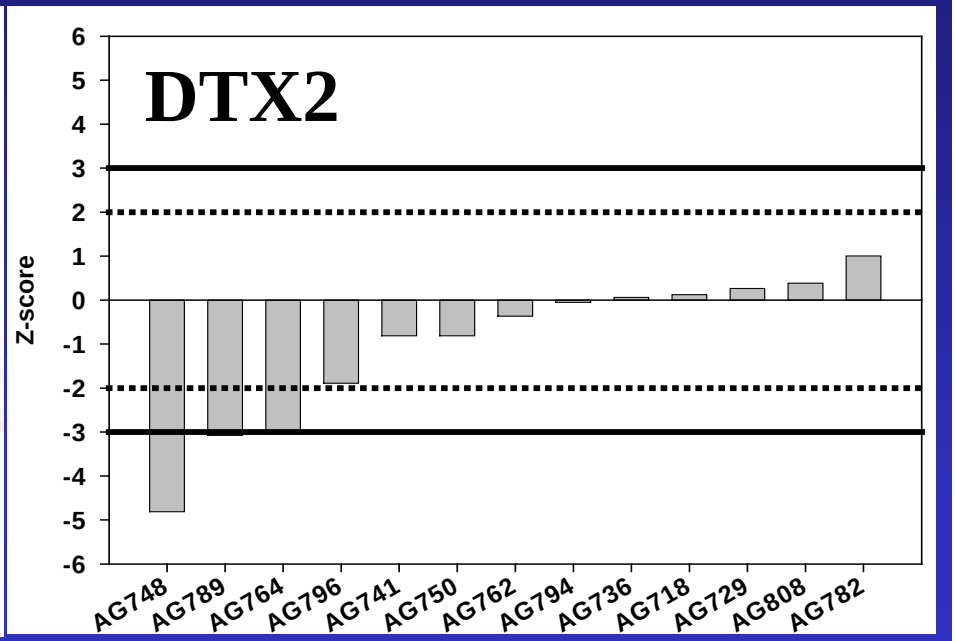
Wet Shellfish Tissue Test Materials for Proficiency Testing

				Homogeneity	Stability			
	Material	Matrix	Analyte	CV (%)	Frozen (-20°C)	+4 °C	+20 °C	+40 °C
DA	1	Scallop Adductor	DA	2.1	30 days	30 days	*n/a*	4 days *
	2	Whole Scallop	DA	3.7	30 days	30 days	16 days	4 days
	3	Whole Mussel	DA	2.7	30 days	30 days	*n/a*	8 days
	4	Oyster	DA	2.1	30 days	30 days	*n/a*	30 days
	5	Clam	DA	2.2	30 days	30 days	*n/a*	4 days *
OA group	6	Whole Scallop	OA	6.2	30 days	30 days	*n/a*	30 days
	7	Oyster	DTX1	6.2	30 days	30 days	16 days	16 days
	8	Mussel	DTX2	4.6	30 days	30 days	30 days	*n/a*
	9	Clam	DTX2	6.3	30 days	30 days	*n/a*	30 days
AZA group	10	Mussel	AZA1	3.1	30 days	30 days	30 days	30 days
	11	Mussel	AZA1	4.9	30 days	30 days	30 days	30 days
						n/a: no data was acquired for this condition		

Materials sufficiently homogenous and stable

Proficiency testing – Essais d'aptitude





Proficiency Tests for DA

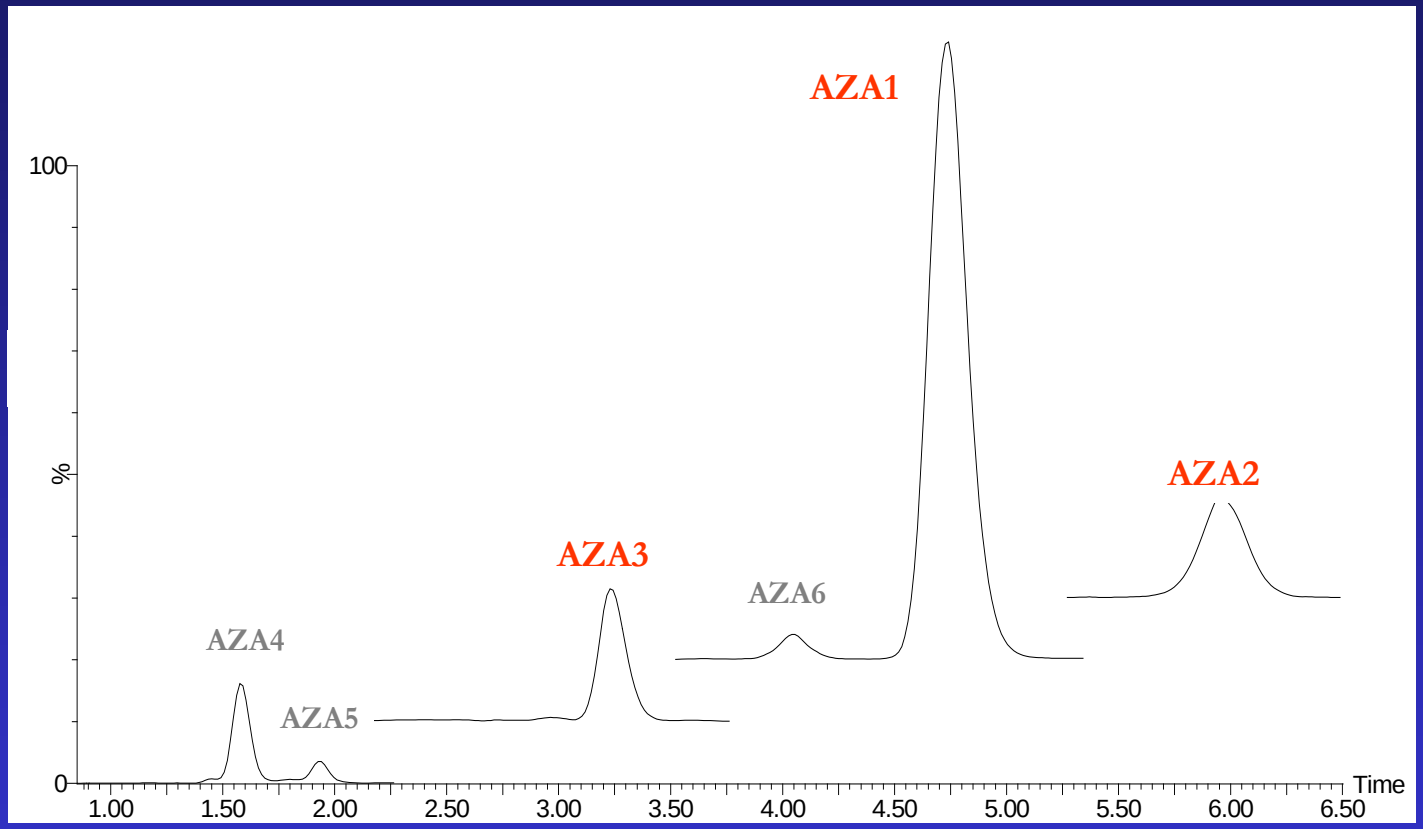
Overview of all 6 exercises within QUASIMEME

Methods used include LC-UV, LC-MS and ELISA

DA	Year	Participants	Returns	100%	>75%	% 100%
DE9-R34	**2003**	40	36	21	28	58
DE9-R36	**2003/4**	42	34	20	27	59
DE9-R39	**2004/5**	38	33	22	25	67
DE9-R41	**2005**	41	38	29	33	76
BT7-R43	**2005/6**	34	31	20	20	65
BT7-R45	**2006**	35	32	27	27	84

→ PT can work for Shellfish Toxins !

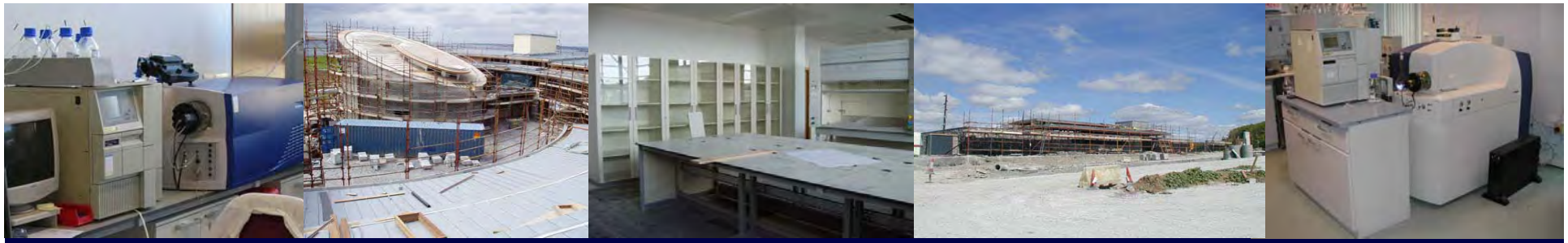
Collaboration with National Research Council of Canada (NRCC) on candidate CRM for AZAs



Acquired on 2795 Waters HPLC coupled to a Micromass Q-TOF Ultima (Z-spray ESI). Isocratic elution on C_{18} ACE (30 mm x 2.1 mm) using 60 % B (Binary mobile phase with A (100 % aqueous) and B (95 % acetonitrile) each containing 2 mM ammonium formate and 50 mM formic acid).

Marine Institute
Foras na Mara


National Research Council Canada
Institute for Marine Biosciences
Conseil national de recherches Canada
l'Institut des biosciences marines
Certified Reference Materials Program
Programme des matériaux de référence certifiés
NRC CRM-AZA-Mus
3639
MUSSEL TISSUE HOMOGENATE/TISSUS DE MOULES
Store at <-12 °C/ Ranger à <-12 °C
Lot No./No de lot: 200603
NRC CRM-
Lot Number
Unit Numb
NRC · CNRC
An International Partnership
NRC CRM-AZA-Mus
Marine Institute



Operational Estimate of AZA1-tissue CRM use

If 50 laboratories use 12 portions / year

- ↳ 600 portions / year total consumption
- ↳ in 5 years, 3000 portions used
- ↳ Current CRM is used up < 5 years

Marine Institute, IRMM and NRCC collaboration on multi-toxin CRM (2007)

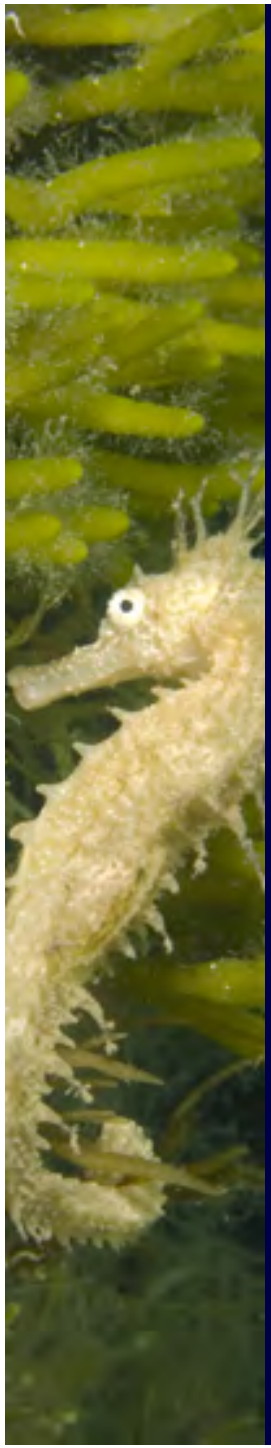
- *MI:* Supply of tissues, composition of material, adjusting concentrations and bulk mixing, initial stabilisation (heat treatment/additives).
- *IRMM:* Adjustment of water contents, freeze-drying, grinding, milling, sieving, packaging, KFT and PSA.
- *NRCC:* Contribution of some materials, toxin characterisation of material, stability studies, certification, storage and distribution.

Composition of multi-toxin material (100 – 150 kg wet)

Material	Country	Institute	Harvest Year	Toxins	Quantity (ca.)
Mussels	Canada	NRCC	1989	DA	4 kg
Mussels	Ireland	MI	2005	AZAs	24 kg
Mussels	Ireland	MI	2004	OA/DTX2 & esters	65 kg
Mussels	Norway	MI	2006	DTX1	7 kg
Algal Paste	Canada	NRCC	n/a	SPX 13 - desMe - C	tbc
Algal Paste			n/a	YTX	tbc
Algal Paste			n/a	PTX2	tbc

Projected toxin concentrations

DA (µg/g)	OA (µg/g)	DTX1 (µg/g)	DTX2 (µg/g)	AZA1 (µg/g)	AZA2 (µg/g)	AZA3 (µg/g)	SPX 13-desMe-C (µg/g)	YTX (µg/g)	PTX2 (µg/g)
25.00	0.33	0.04	0.62	0.74	0.18	0.18	0.30	0.5	0.1



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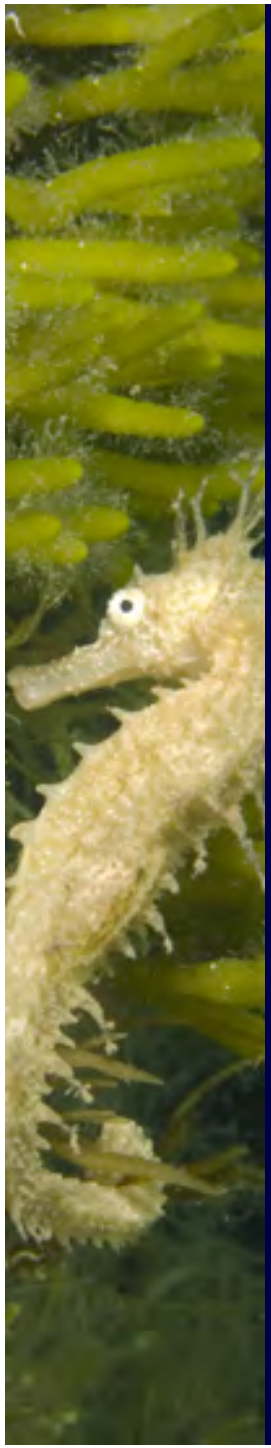
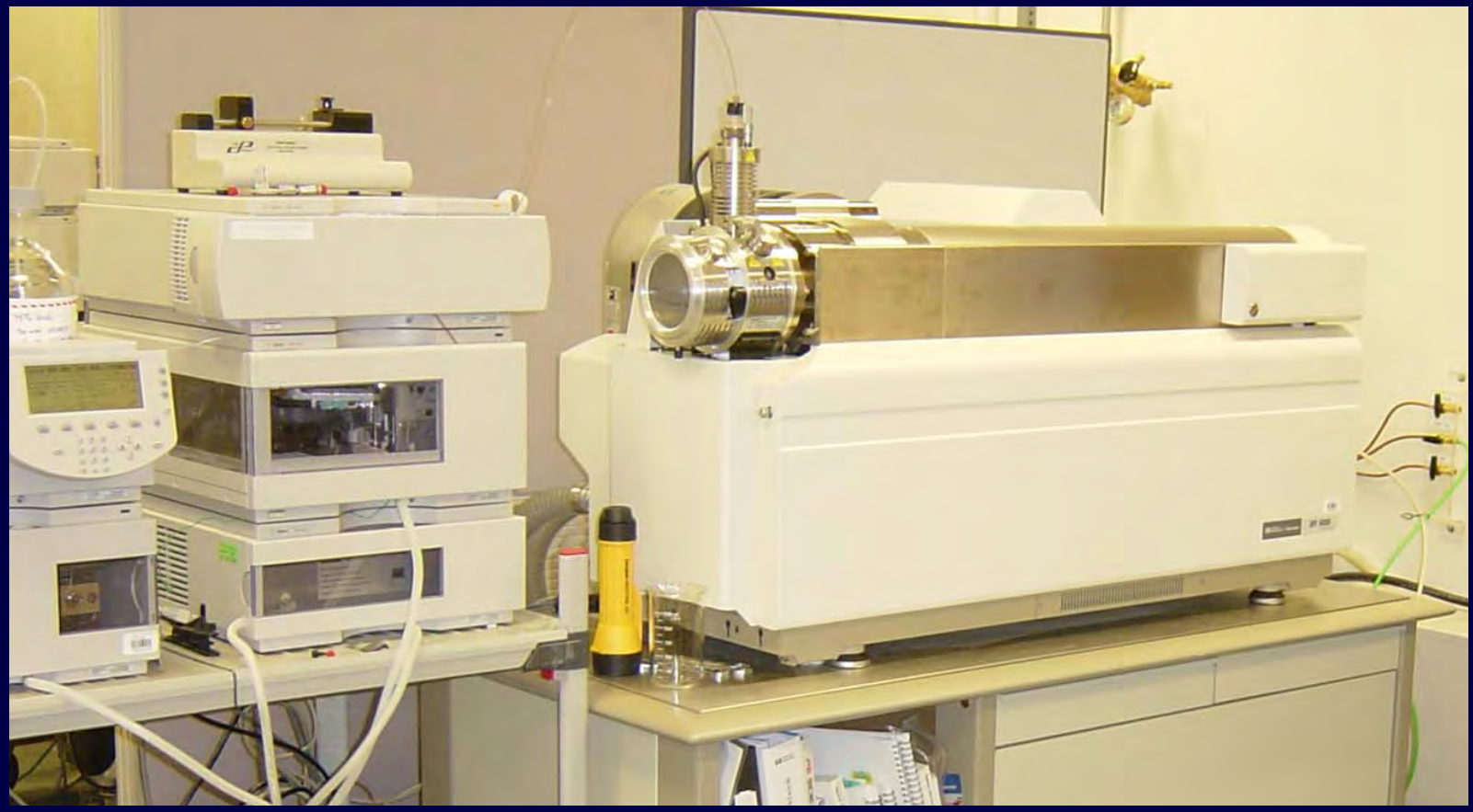
- ✓ **Comparison of detection techniques**
- ✓ Characterisation of toxin hazards
- ✓ Outlook

Biological detection methods



Physico-chemical analytical Methods



MBA for AZAs –

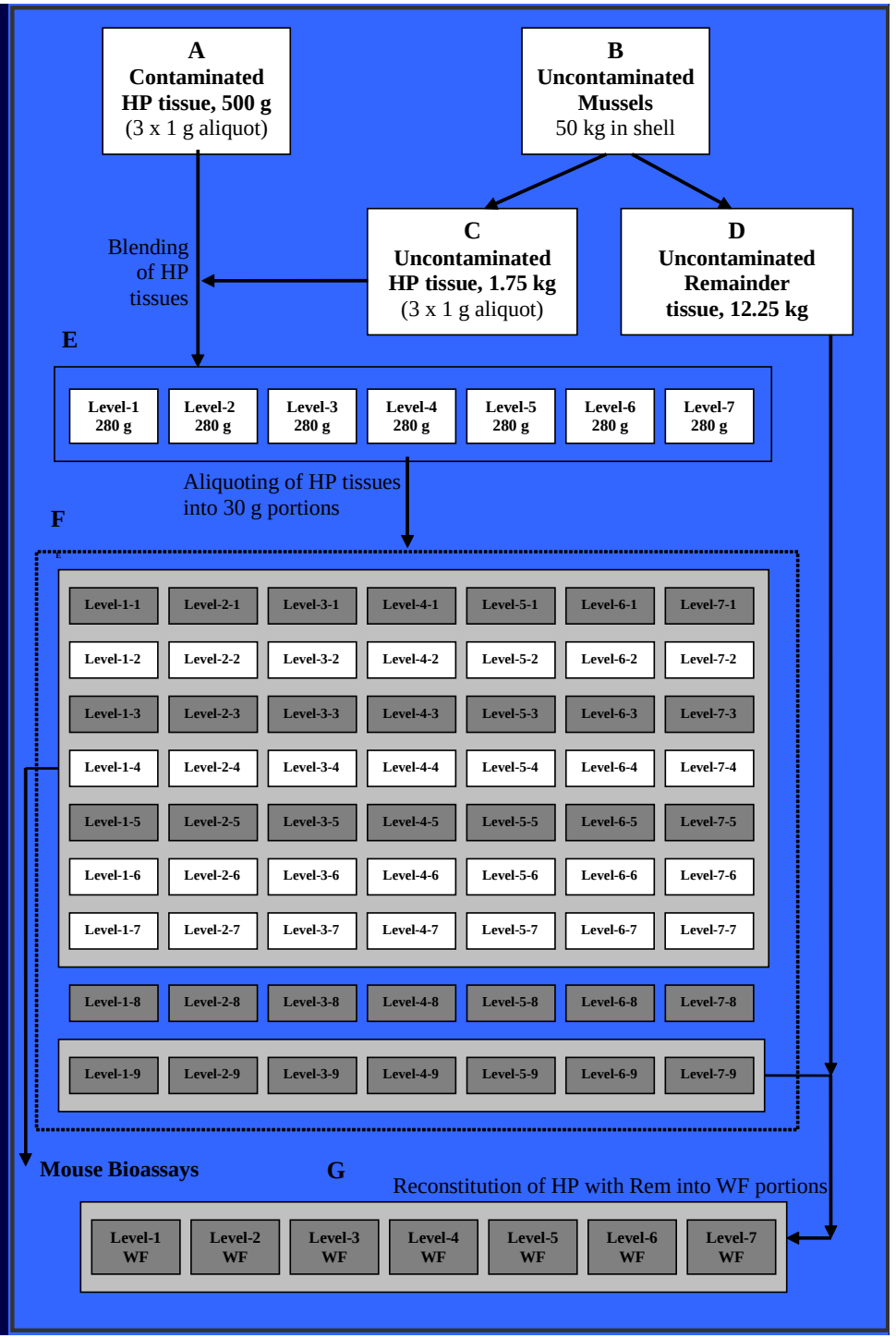
Study design

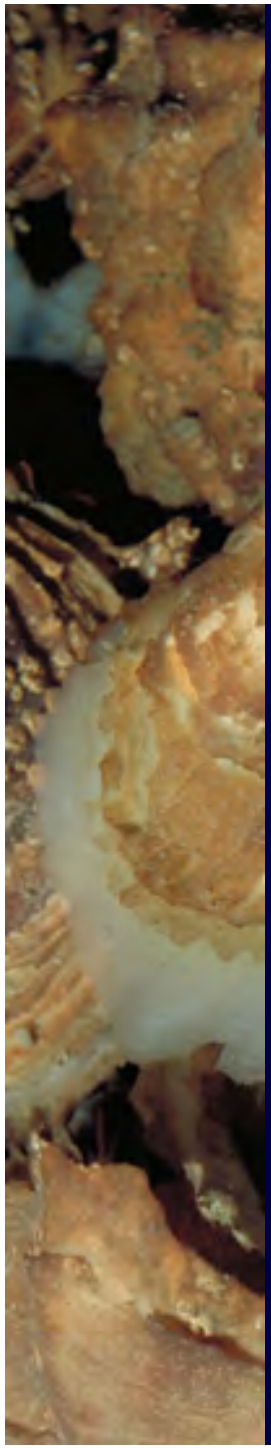
Tissue dissections and mixtures

Tissue A:
Mussels from southwest Ireland
Autumn 2007, AZA-only event

Tissue B:
Mussels from east coast of Ireland
Typically AZA-"free" production zone

Hess et al., *Toxicon* 2009, 53, 713-722.





Homogeneity

Table 1. Homogeneity study. Relative standard deviations (RSDs) in [%], n=5.

Level	1	2	3	4	5	6	7
RSD	n/a*	3.5	1.8	5.2	6.6	4.0	4.6

*n/a = RSD is not applicable where concentrations were not quantifyable

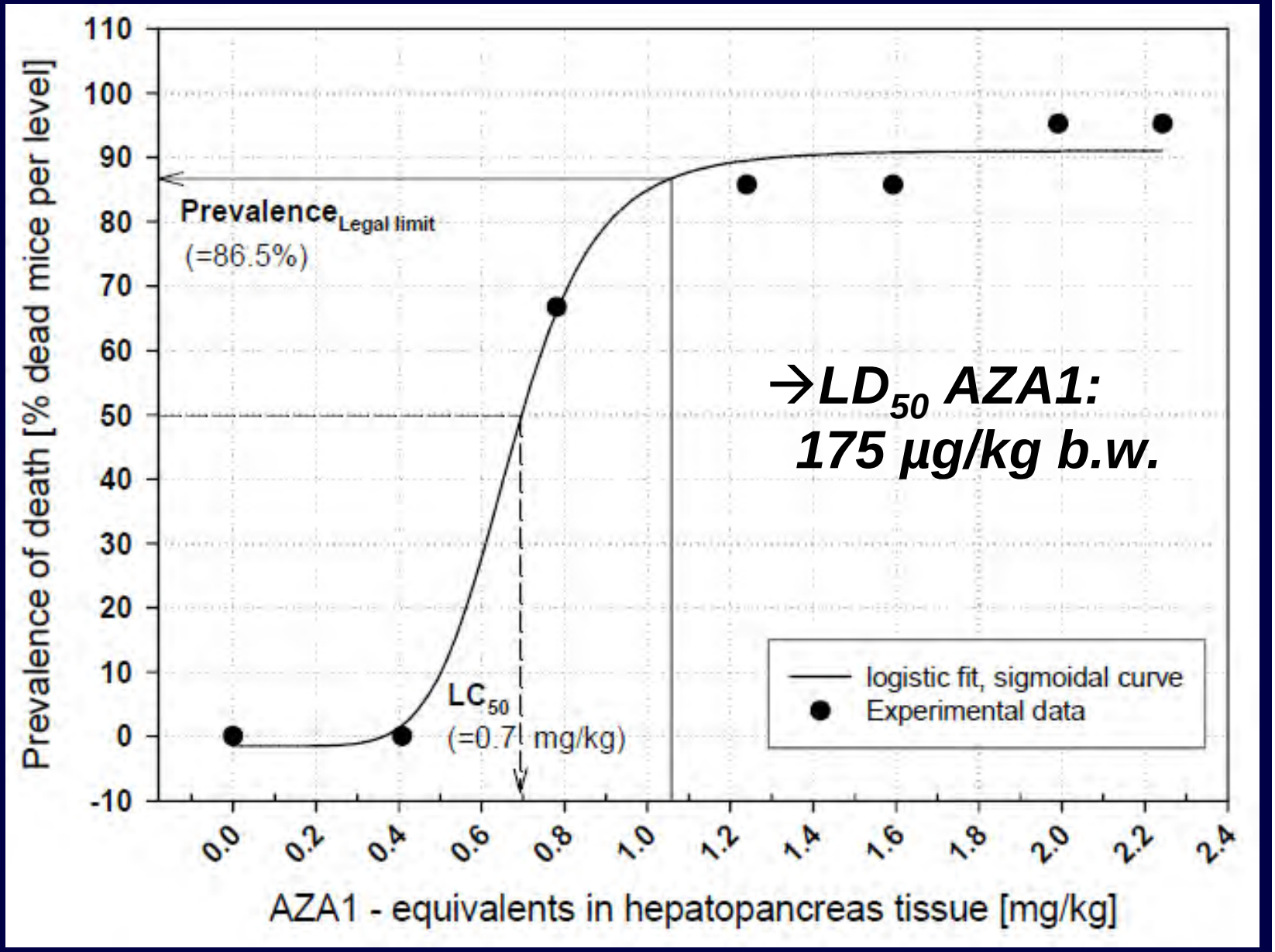
Accuracy of assigned values

Table 2. AZA1-equivalents determined in mussel hepatopancreas (HP) and whole flesh (WF) homogenates. All concentrations expressed in whole flesh AZA1-equivalents [mg/kg]*, n=3. Sample-to-solvent ratios were 1 : 62.5 (diluted HP extracts), 1 : 12.5 (crude HP extracts) and 1 : 12.5 (WF extracts).

Level	1	2	3	4	5	6	7
AZA1-Equiv. (dilute HP extracts)	**<LOQ**	**0.06**	**0.12**	**0.19**	**0.24**	**0.30**	**0.34**
AZA1-Equiv. (crude HP extracts)	<LOQ	0.05	0.09	0.13	0.17	0.20	0.23
AZA1-Equiv. (reconst. WF extracts)	<LOQ	0.06	0.11	0.15	0.19	0.26	0.32

*An average HP content of 15.25% was used for transformation of the AZA1-equivalents determined in HP into whole flesh equivalents. As the reconstituted whole flesh samples had an actual HP content of 20%, the concentrations were also back-calculated to reflect the average HP content of 15.25%.

Concentration – lethality response curve

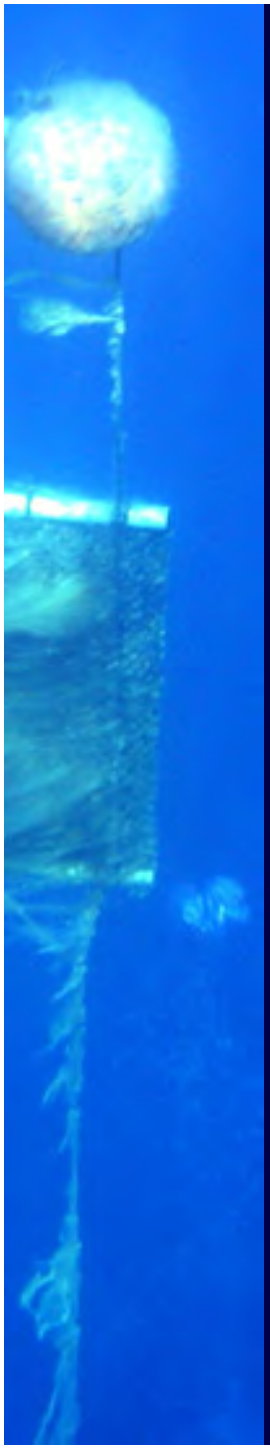


Mouse Bioassay
Detection Capability expressed as Probability of Detection of Positive at regulatory limit

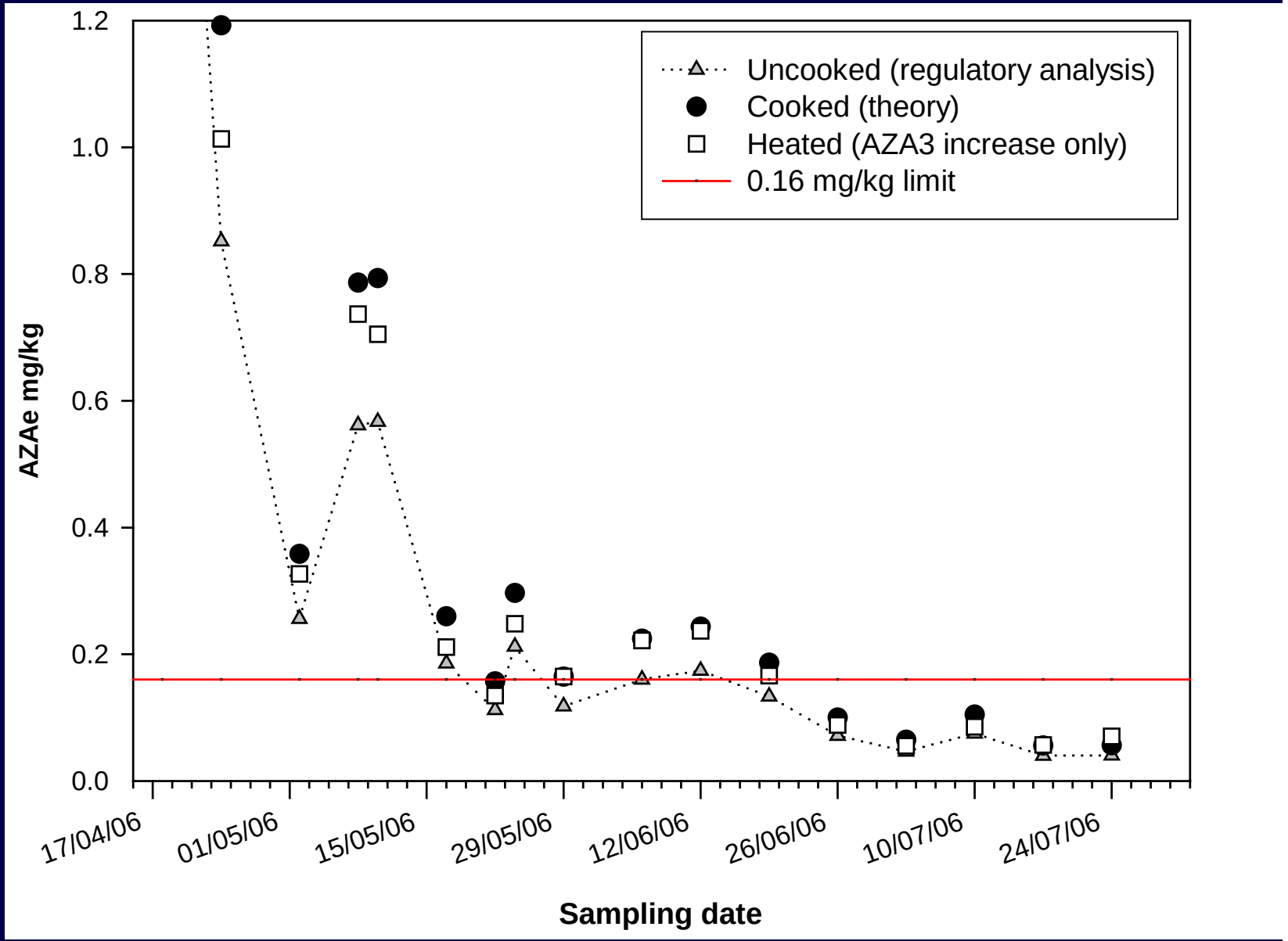
Table 5. Calculation of the probability (Pr) of a positive (pos) or negative (neg) mouse bioassay for shellfish at the current regulatory limit (0.16 mg/kg). A mouse bioassay is positive when 2 or 3 mice are dead after 24 h observation.

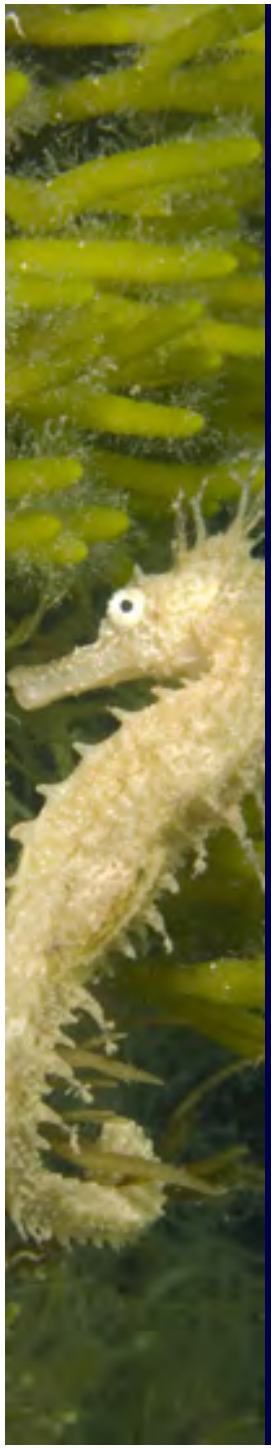
mouse	mouse	mouse	Pr of individual			Pr (scenario)		
1	2	3	mouse to live or die					
dead	dead	dead	0.865	0.865	0.865	0.647		
dead	dead	alive	0.865	0.865	0.135	0.101		
dead	alive	dead	0.865	0.135	0.865	0.101		
alive	dead	dead	0.135	0.865	0.865	0.101	Pr(pos)	0.95
dead	alive	alive	0.865	0.135	0.135	0.016		
alive	dead	alive	0.135	0.865	0.135	0.016		
alive	alive	dead	0.135	0.135	0.865	0.016		
alive	alive	alive	0.135	0.135	0.135	0.002	Pr(neg)	0.05
					Total	1.00		

@ RL of 80 µg/kg, the probability of detecting a positive decreases to 5% !



Natural detoxification of mussels and processing





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Stability of AZA to acids in solution

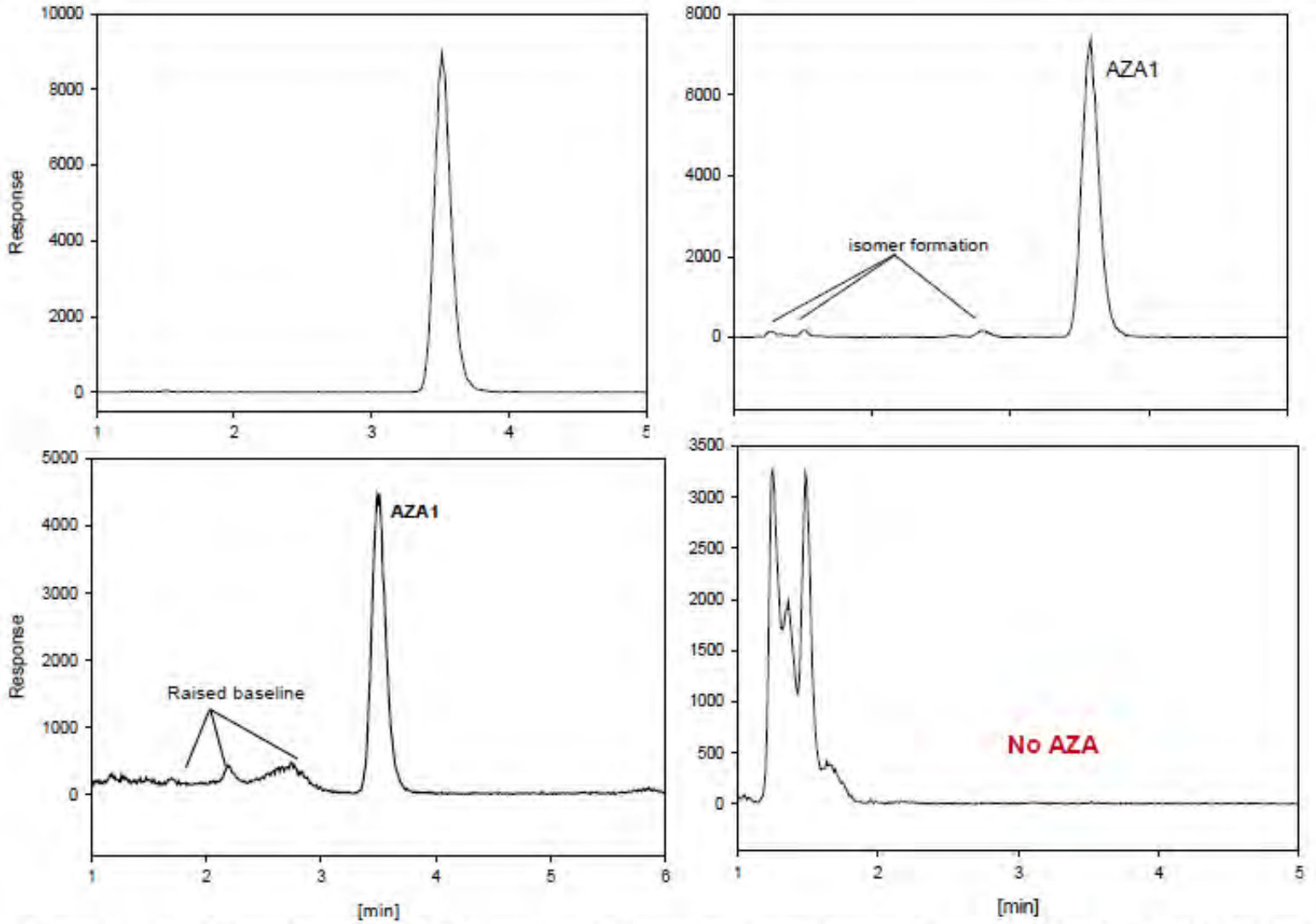
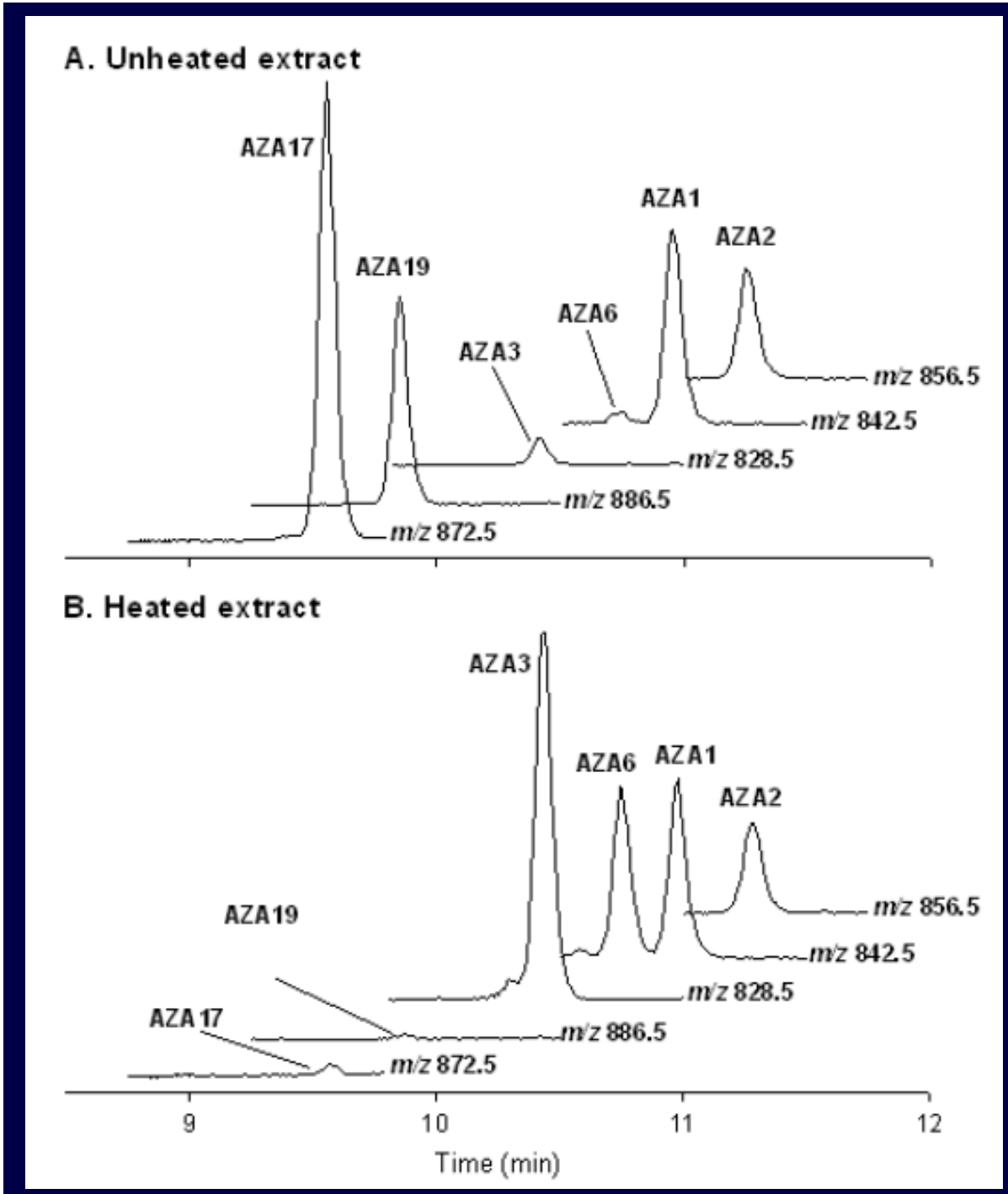


Figure 6.1.3. Degradation of AZA1 under different pH conditions: pure standard in MeOH kept for 48 h at 45°C (top left), standard in aqueous acetonitrile exposed to acetic acid (0.1 %) for 48 h at 45°C (top right), standard in aqueous acetonitrile exposed to NaOH (50 mM) for 24 h at room temperature (bottom left), standard in aqueous acetonitrile exposed to formic acid for 48 h at 45°C (bottom right).

Alfonso C., Rehmann N., Hess P., Alfonso A., Wandscheer C., Abuin M., Vale C., Otero P., Vieytes M., Botana L.M. (2008) Anal. Chem., 80 (24) 9672-9680.



Stability of carboxy-AZAs to heat

AZA-group toxins increase by 30 - 100 % in processing !

Hess et al., 2005
***Toxicon* 46, 62-71**

Stability of carboxy-AZAs to heat

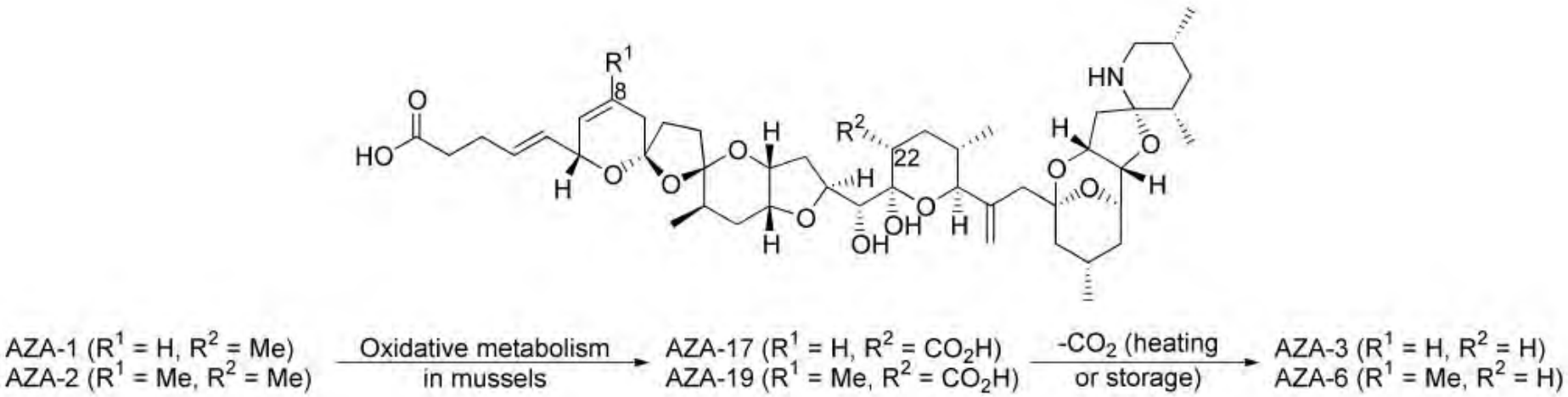


Figure 6.1.12. Proposed oxidative metabolism of AZA1 and -2 in shellfish to form 22-carboxylated metabolites (AZA17 and -19), which undergo decarboxylation when heated to form AZA3 and -6, respectively, adapted from McCarron et al., 2009.

McCarron et al., 2009 *J. Agric. Food Chem*. 57, 160-169.

Acid-constants of some toxins

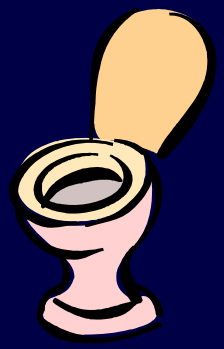
Toxin	Predicted pK_a	Experimental pK_a
AZA1	4.90, 9.20	5.8 ± 0.2
AZA2	4.90, 9.20	5.8 ± 0.2
OA	3.80	4.9 ± 0.5
DTX1	3.80	4.9 ± 0.5
DTX2	3.80	4.9 ± 0.5
PTX2	No acidic function	No apparent pK_a observed
YTX	na	6.9 ± 0.5

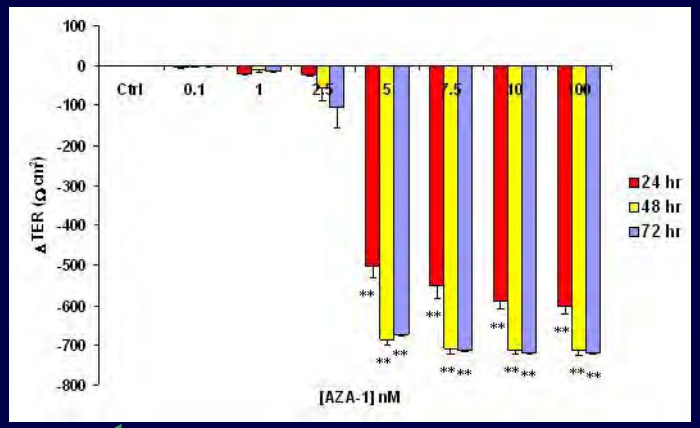
Lipophilicity of some toxins

Toxin	Predicted $logP_{ow}$	Experimental $logP_{ow}$
AZA1	4.06 ± 2.20	7.54 ± 1.01
AZA2	4.32 ± 2.33	8.18 ± 1.28
OA	4.45 ± 2.60	5.05 ± 0.72
DTX1	4.79 ± 2.67	6.88 ± 1.41
DTX2	4.46 ± 2.55	5.61 ± 0.81
PTX2	4.45 ± 1.99	6.47 ± 2.37

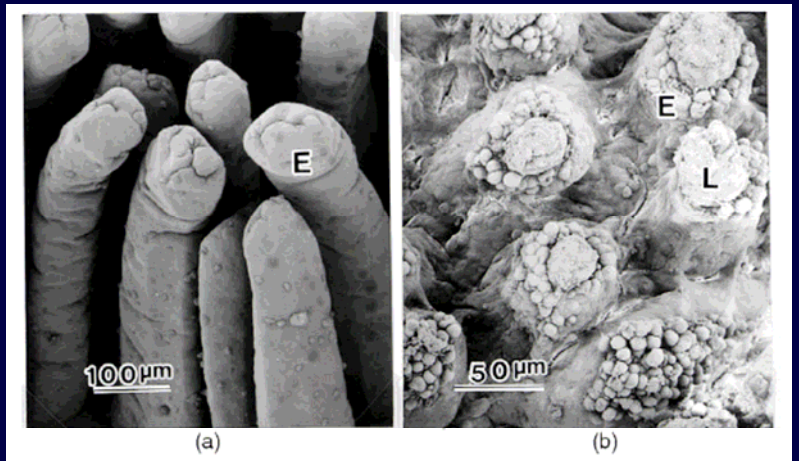
Characterising AZA - toxicity

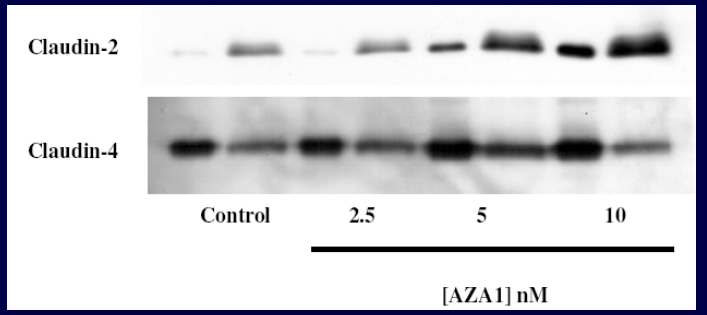
A lot of symptoms and findings from *in-vivo* & *in vitro* studies overlap:





Upregulation in caco-2 cells of genes involved in wound-healing

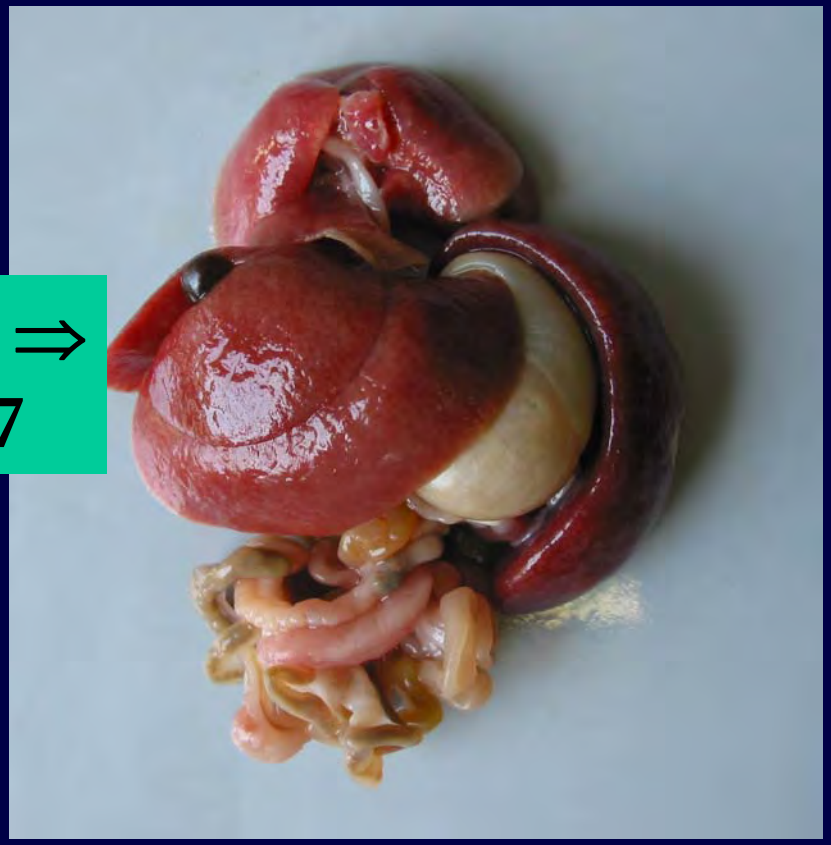




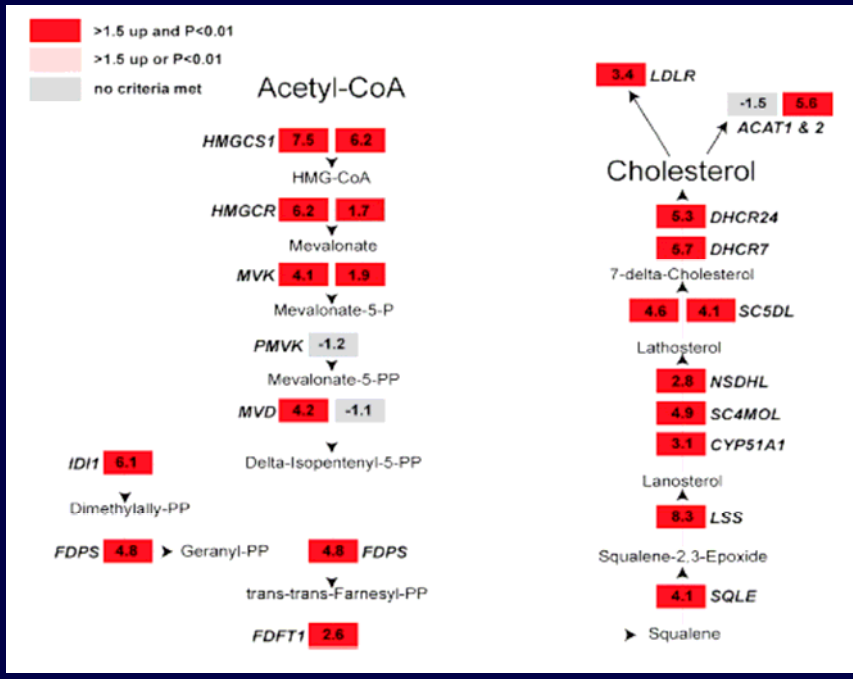
Characterising AZA - toxicity - 2

A lot of symptoms and findings from *in-vivo* & *in vitro* studies overlap:

Liver ⇒
X 2.37



Lipid-metabolism path



Relative toxicity of DTX2 compared to OA in mice

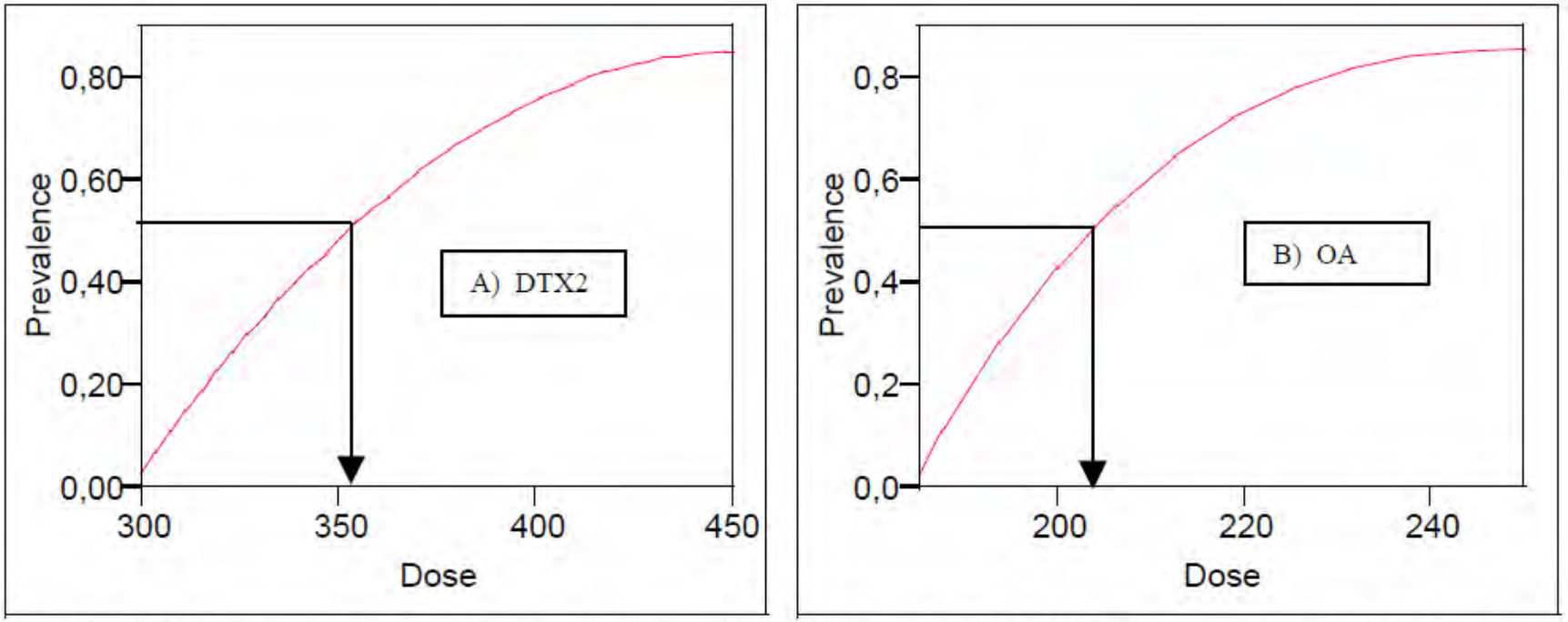
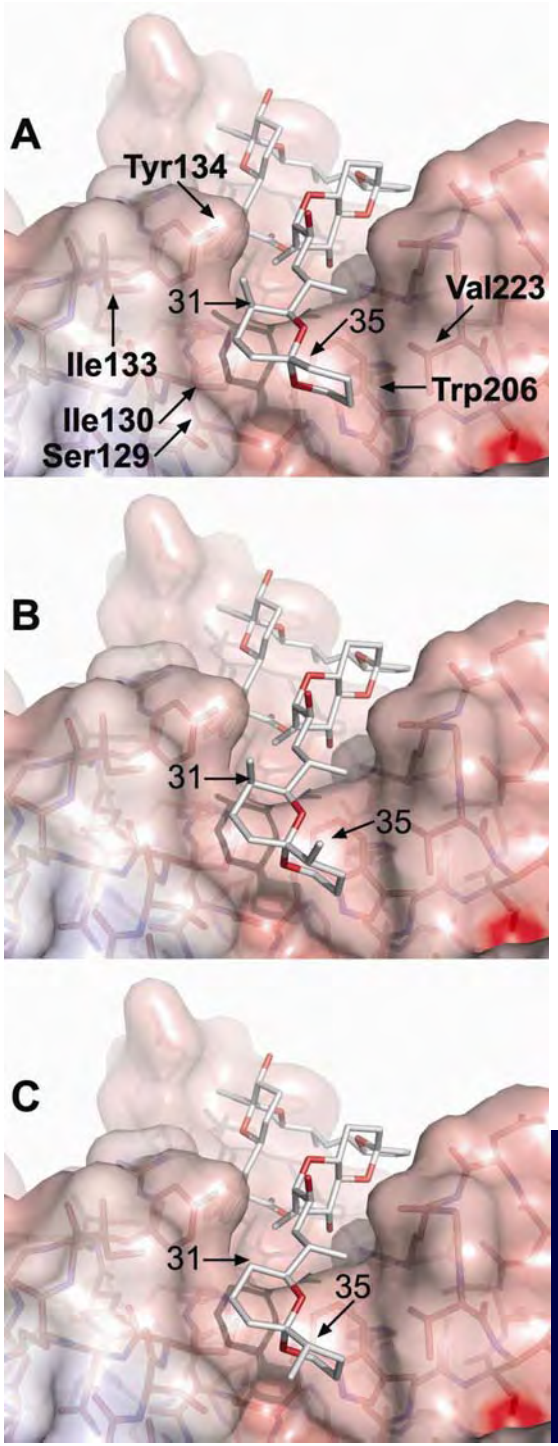
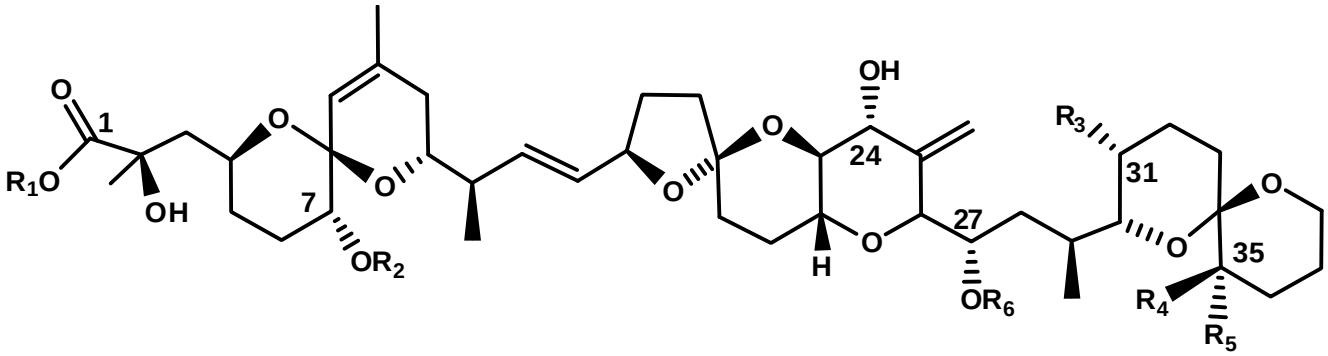


Figure 6.3.1. The prevalence of death as a function of A) DTX2 dose and B) OA dose [µg], as predicted by the use of a second degree polynome.

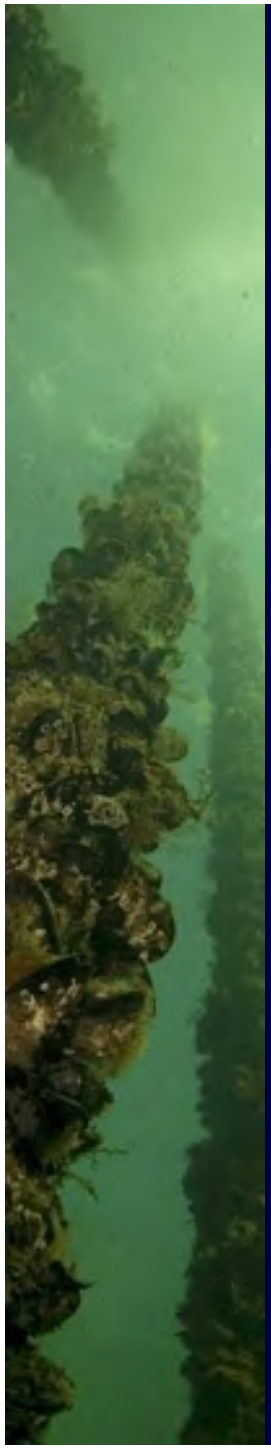
Aune T, Larsen S., Aasen J., Rehmann N., Satake M. and Hess P. (**2007**) Relative toxicity of dinophysistoxin-2 (DTX-2) compared with okadaic acid, based on acute intraperitoneal toxicity in mice. *Toxicon* 49 1-7.





	R1	R2	R3	R4	R5	R6
OA	H	H	CH3	H	H	H
DTX1	H	H	CH3	CH3	H	H
DTX2	H	H	H	H	CH3	H
DTX3	H	acyl	as parent	as parent	as parent	H
Diol-ester	diol	H/acyl	as parent	as parent	as parent	H
DTX4	Diol-ester	H	as parent	as parent	as parent	H
DTX5	Diol-ester	H	as parent	as parent	as parent	H
27-O-acyl	H	H	CH3	H	H	acyl

Molecular models of okadaic acid analogues docked to protein phosphatase-1 (PP1), based on the crystal structure of okadaic acid bound toPP1 (PDBid:1JK7). A, okadaic acid; B, dinophysistoxin-1; C, dinophysistoxin-2. Adapted from Larsen et al., 2007



International Expert Consultation - Risk Evaluation

European Food Safety Authority

The EFSA Journal (2009) 1306, 1-23

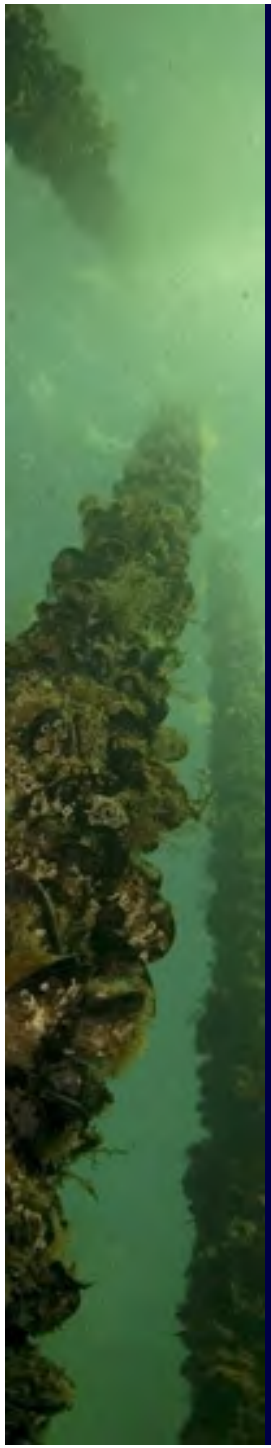
SCIENTIFIC OPINION

Marine biotoxins in shellfish – Summary on regulated marine biotoxins[1]

Scientific Opinion of the Panel on Contaminants in the Food Chain

(Question No EFSA-Q-2009-00685)

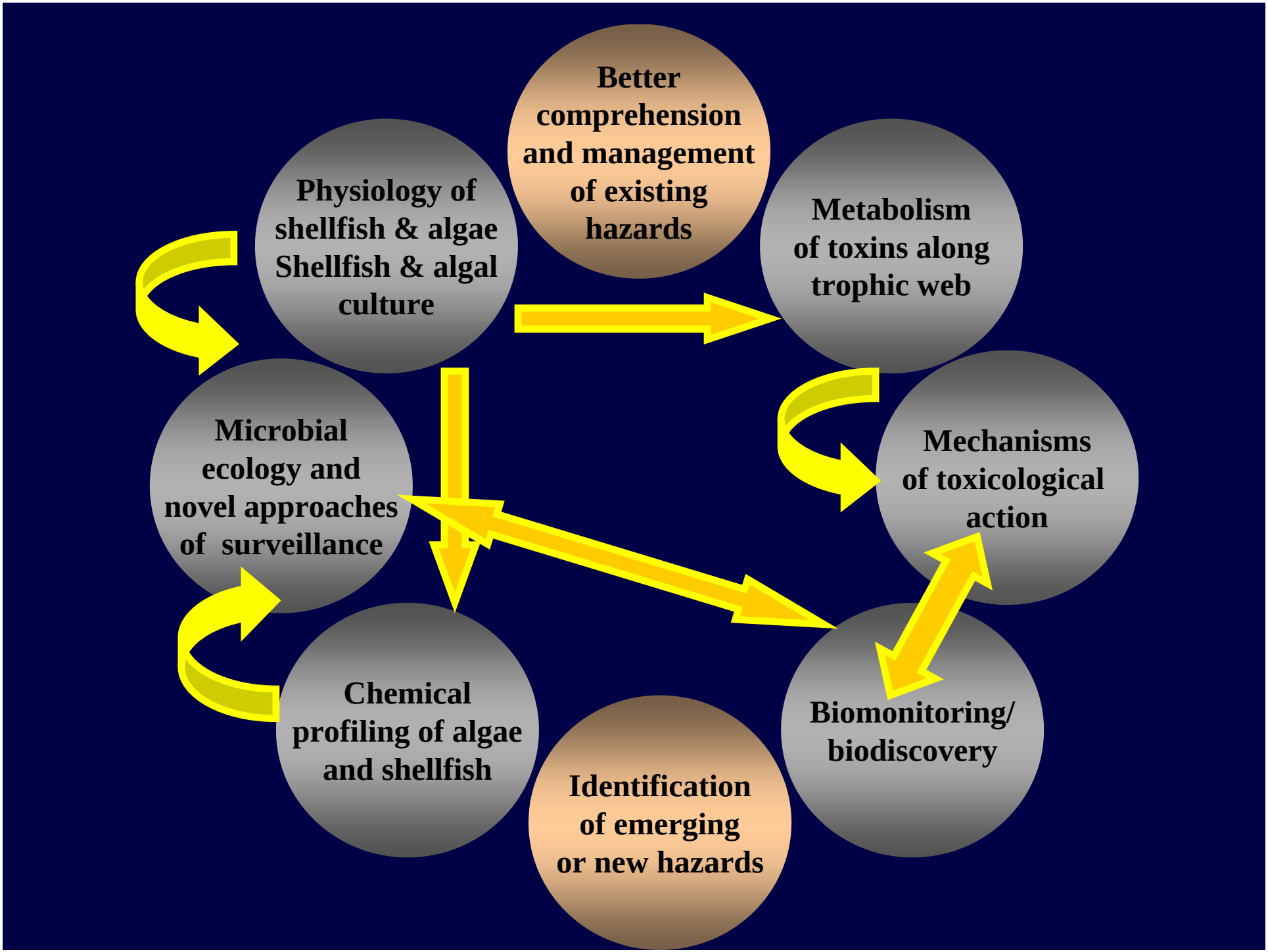
Adopted on 13 August 2009

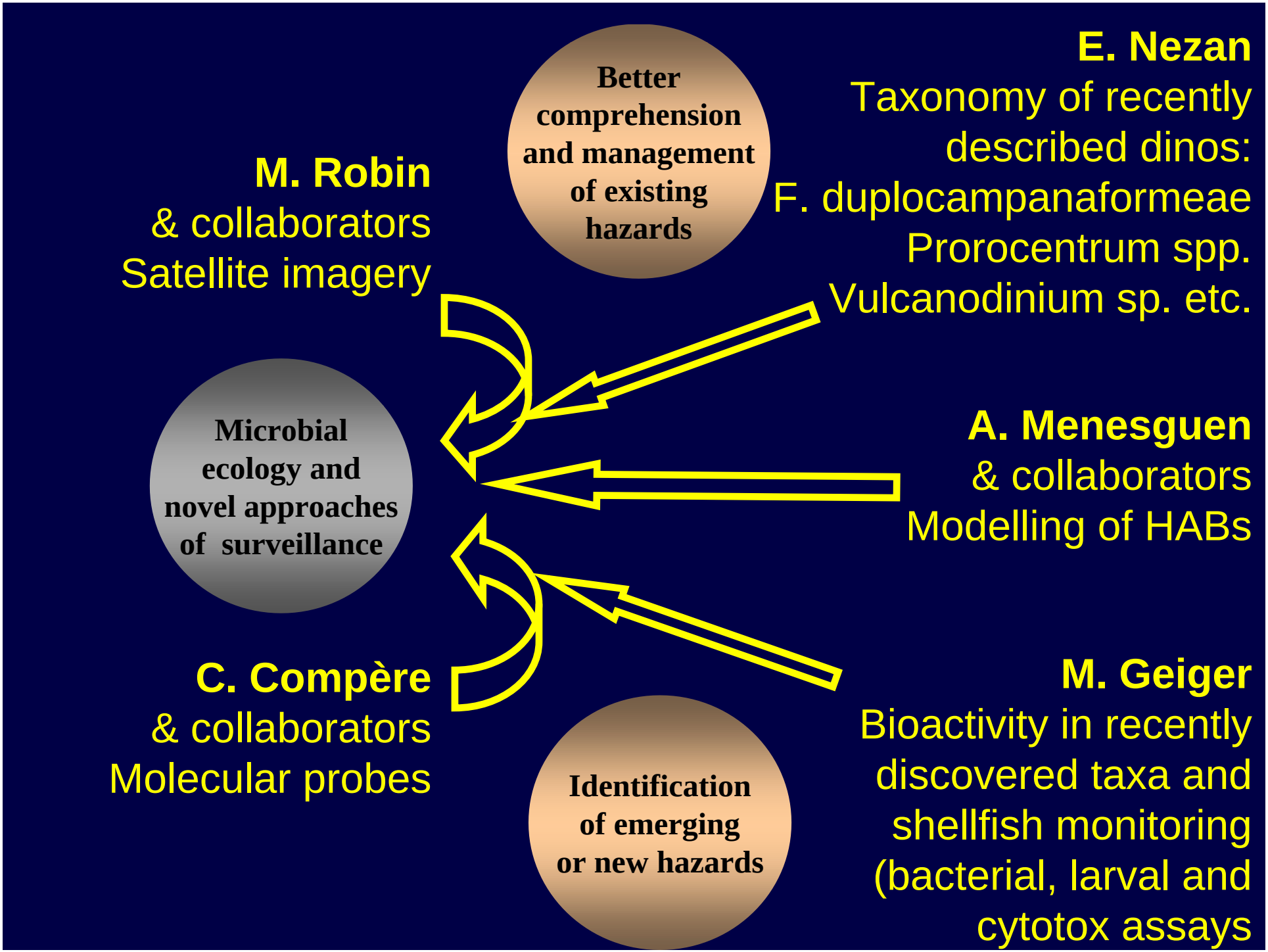


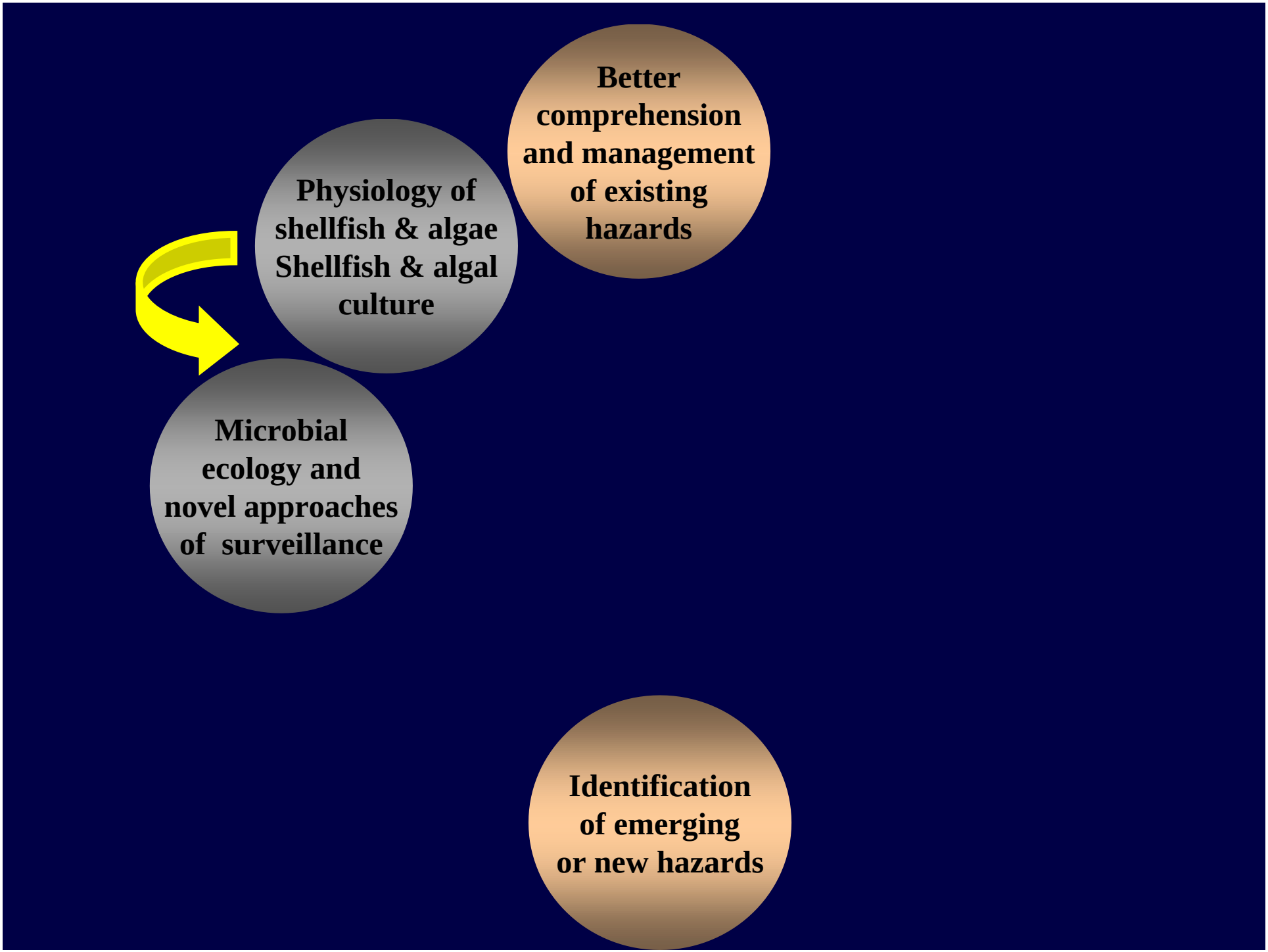
Summary - 1

- ✓ An array of studies have led to improvements in the chemical analysis of phycotoxins, notably domoic acid, saxitoxins and lipophilic toxins. Particular progress was made in the area of azaspiracids where certified calibrants, reference materials and proficiency testing have been made available to scientists and risk managers in this domain.
- ✓ Efforts in surveillance, method validation and several large scale risk evaluation exercises have contributed to a change in legislation for domoic acid and lipophilic toxins.
- ✓ The understanding of metabolism and chemical stability of toxins in shellfish has been addressed for the azaspiracid and pectenotoxin groups. This knowledge has been translated into recommendations for risk managers and the shellfish industry.


Outlook
Better comprehension and management of existing hazards
Metabolism of toxins along trophic web
Mechanisms of toxicological action
Biomonitoring/ biodiscovery
Identification of emerging or new hazards
Chemical profiling of algae and shellfish
Microbial ecology & taxon./ novel approaches of surveillance
Physiology of shellfish & algae shellfish & algal culture


Better comprehension and management of existing hazards
Physiology of shellfish & algae Shellfish & algal culture
Metabolism of toxins along trophic web
Microbial ecology and novel approaches of surveillance
Mechanisms of toxicological action
Chemical profiling of algae and shellfish
Identification of emerging or new hazards
Biomonitoring/ biodiscovery


Better comprehension and management of existing hazards
E. Nezan
Taxonomy of recently described dinos:
F. duplocampanaformeae
Prorocentrum spp.
Vulcanodinium sp. etc.
M. Robin
& collaborators
Satellite imagery
Microbial ecology and novel approaches of surveillance
A. Menesguen
& collaborators
Modelling of HABs
C. Compère
& collaborators
Molecular probes
Identification of emerging or new hazards
M. Geiger
Bioactivity in recently discovered taxa and shellfish monitoring (bacterial, larval and cytotox assays


Better comprehension and management of existing hazards
Physiology of shellfish & algae Shellfish & algal culture
Microbial ecology and novel approaches of surveillance
Identification of emerging or new hazards

ASTOX2 – PhD:
Physiology of *Azadinium spinosum*
-
Thierry Jauffrais

Supervisors: C. Herrenknecht, V. Séchet, L. Barillé

Physiology of A. spinosum

Recovery of cells

Production of toxic biomass

Isolation of toxins

Accumulation of toxins in shellfish

Culture optimisation and recovery of cells

Large-scale bioreactor:

Maintained for several weeks

Tangential filtration

over 500L of culture harvested

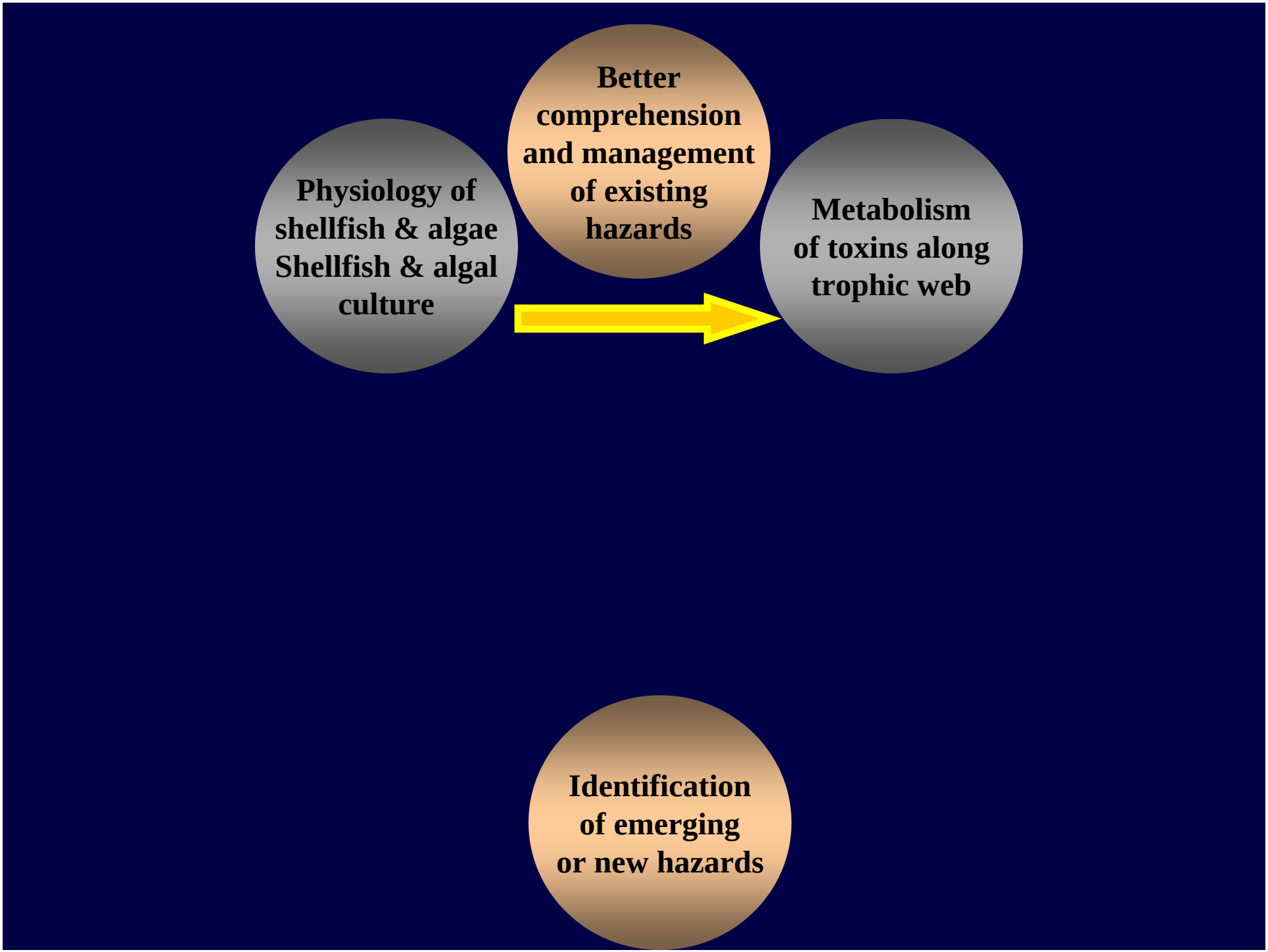
Toxic biomass for isolation of toxins

Large-scale bioreactor:

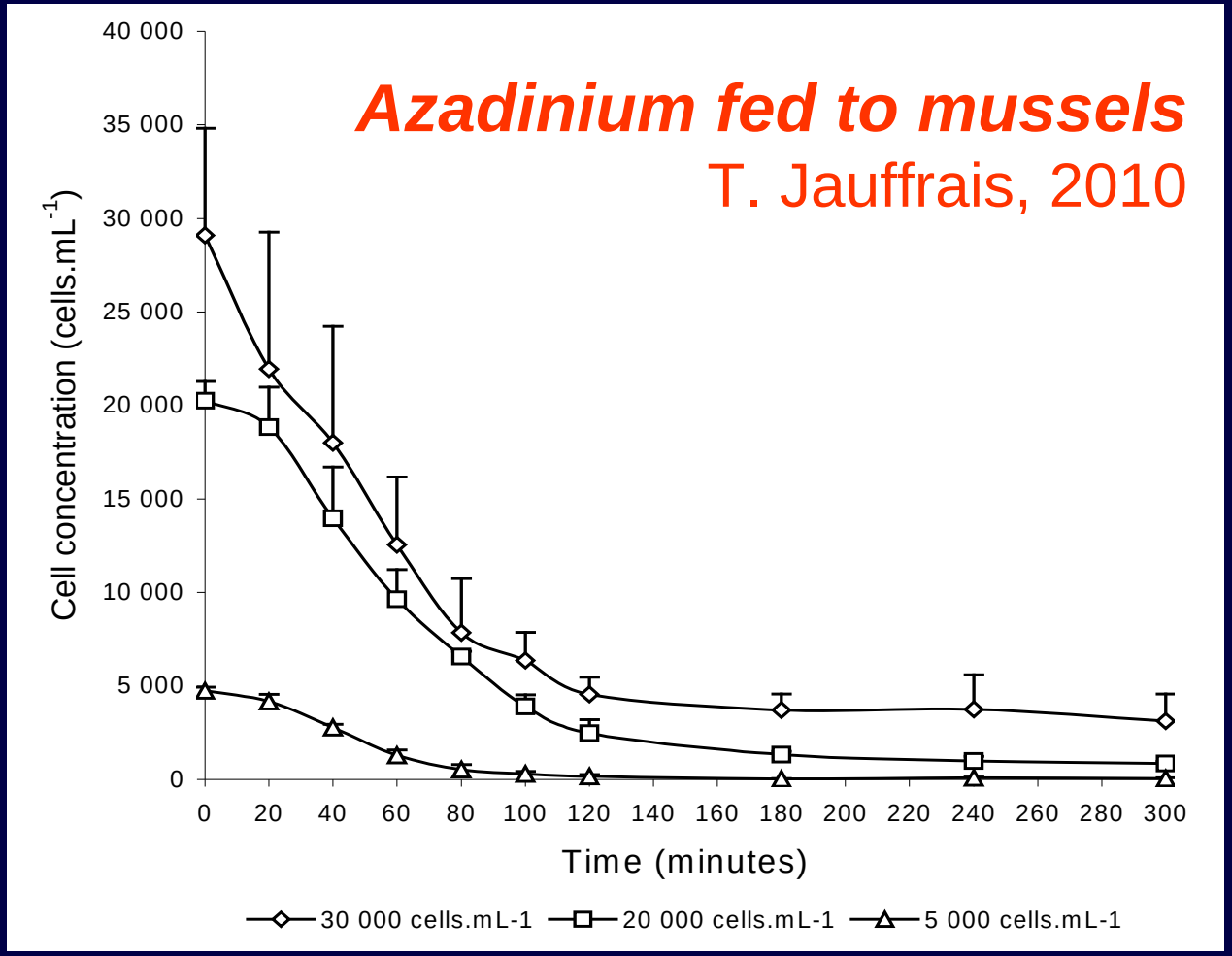
improvement of factor 5 compared to initially reported toxin quota per cell

Currently > 600 L culture harvested → ca. 100 g pellet !

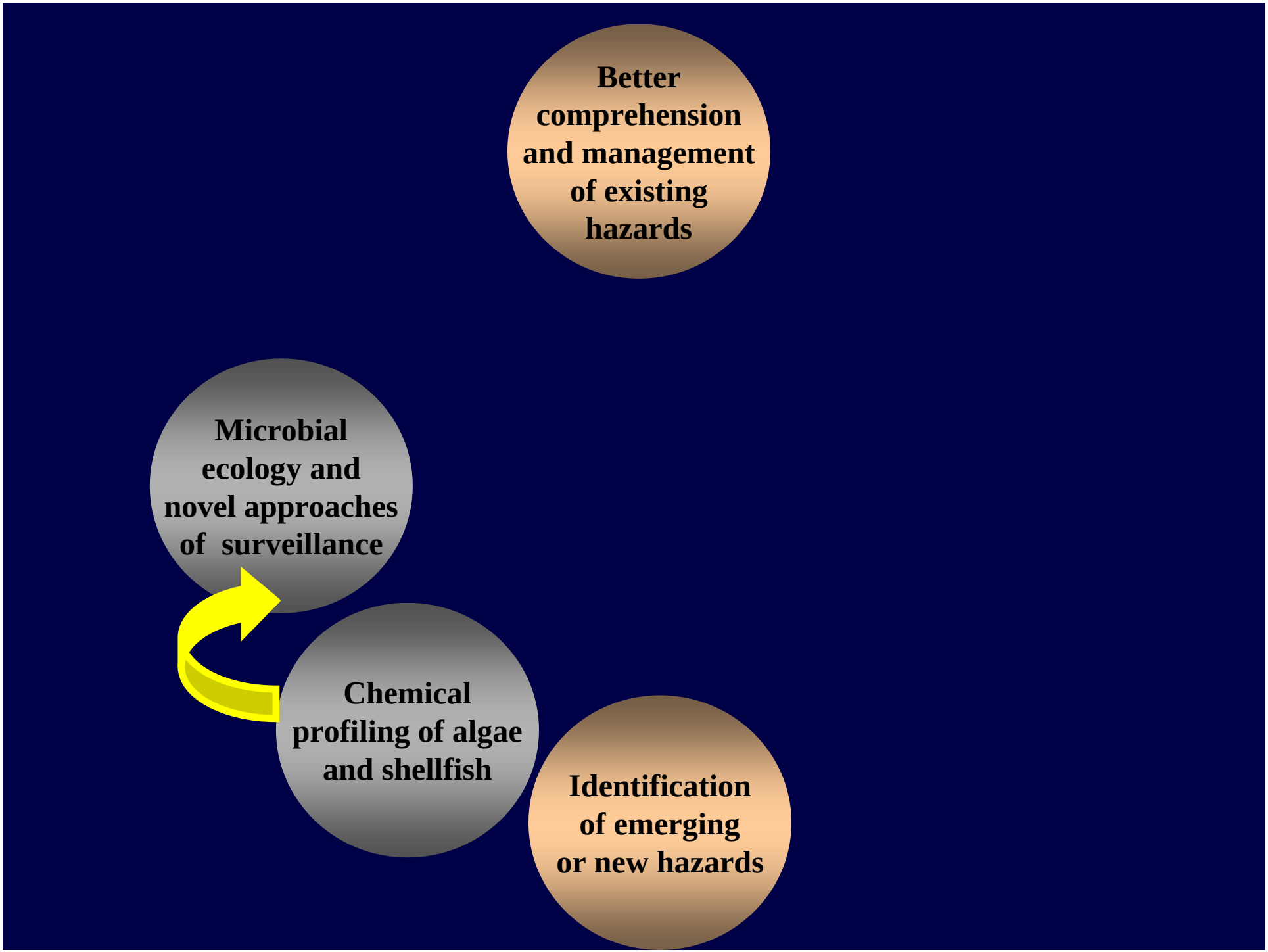
Isolation from this material should give better recovery thanks to higher toxin : matrix – ratio !!


Physiology of shellfish & algae
Shellfish & algal culture
Better comprehension and management of existing hazards
Metabolism of toxins along trophic web
Identification of emerging or new hazards

Accumulation along trophic web



→ **Mussels love Azadinium !!**


Better comprehension and management of existing hazards
Microbial ecology and novel approaches of surveillance
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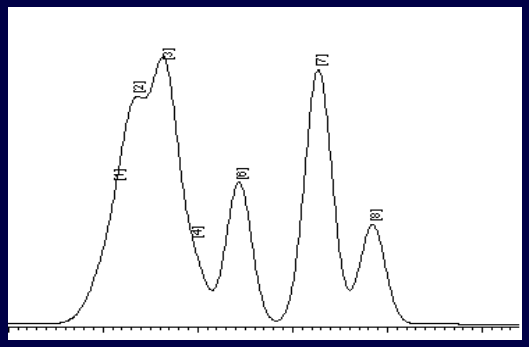
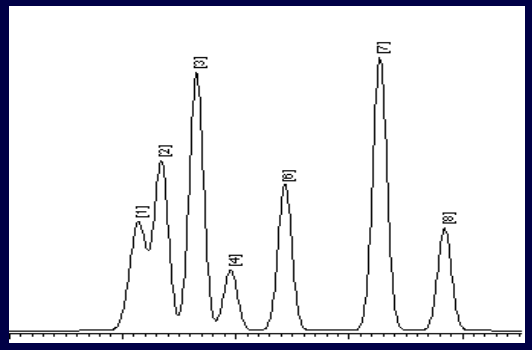
Particle Size Evolution



10 min

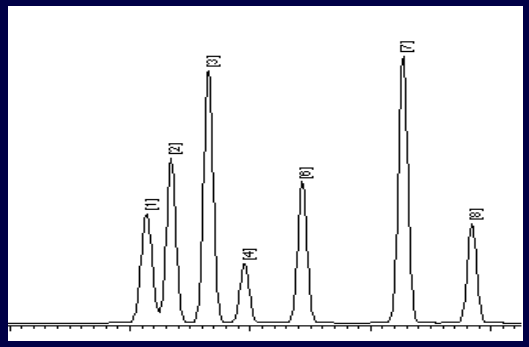
Late 1960's
40µm pellicular non-porous coated
100-500 psi (7-40 bar)
10,000 plates/meter



10 min

Early 1970's
10µm Irregular micro-porous
1000-2500 psi (70-180 bar)
40,000 plates/meter

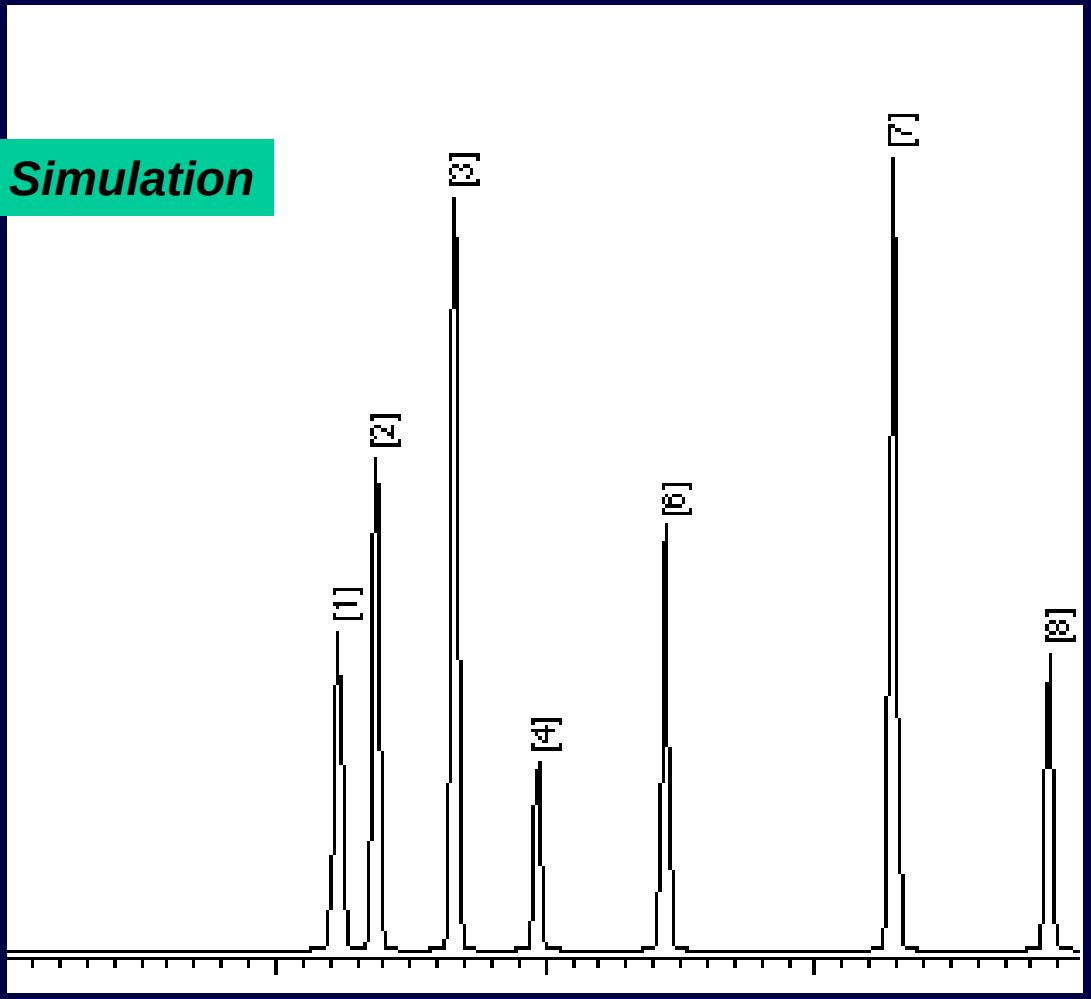
1980's to present day
3.5 - 5µm spherical micro-porous
1500-4000 psi (110-280 bar)
80,000 - 115,000 plates/meter



10 min

Particle Size Evolution

Simulation



1 minute

Future

1.7 µm hybrid particle
2.1 x 100 mm
up to 15,000 psi (1064 bar)

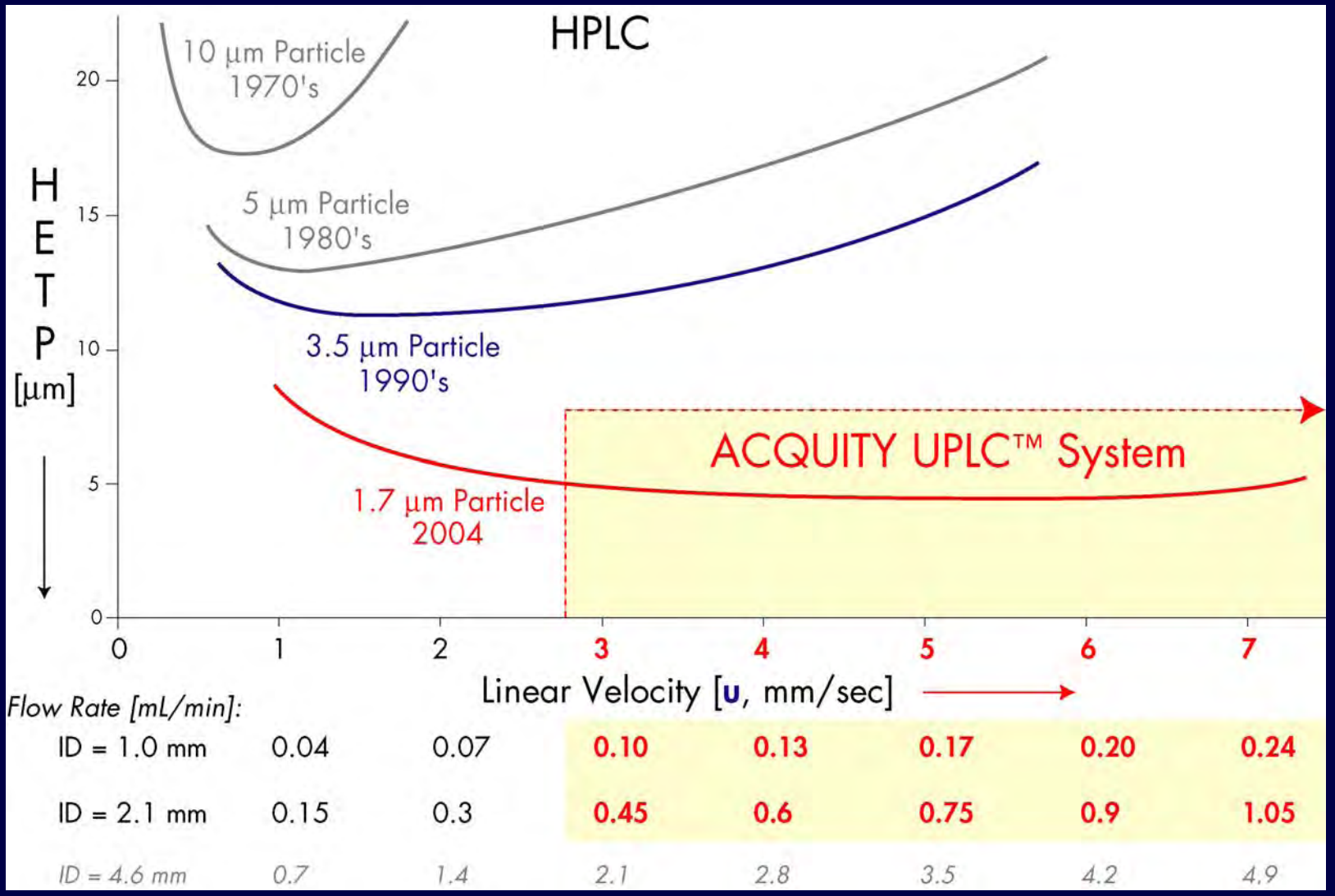
235,000 plates/meter

Combining
Speed and Resolution

Smaller Particles

The enabler of productivity

The promise of the van Deemter plot



Flow Rate [mL/min]:							
ID = 1.0 mm	0.04	0.07	0.10	0.13	0.17	0.20	0.24
ID = 2.1 mm	0.15	0.3	0.45	0.6	0.75	0.9	1.05
ID = 4.6 mm	*0.7*	*1.4*	*2.1*	*2.8*	*3.5*	*4.2*	*4.9*

Analysis of the lipophilic biotoxins by UPLC-MS

HPLC-QTof

Conditions:

BDS Hypersil C8 (50×2mm, 3µm)

2mM ammonium formate, 50mM formic acid

Gradient conditions

Flow rate 0.2mL/min

UPLC-QqQ

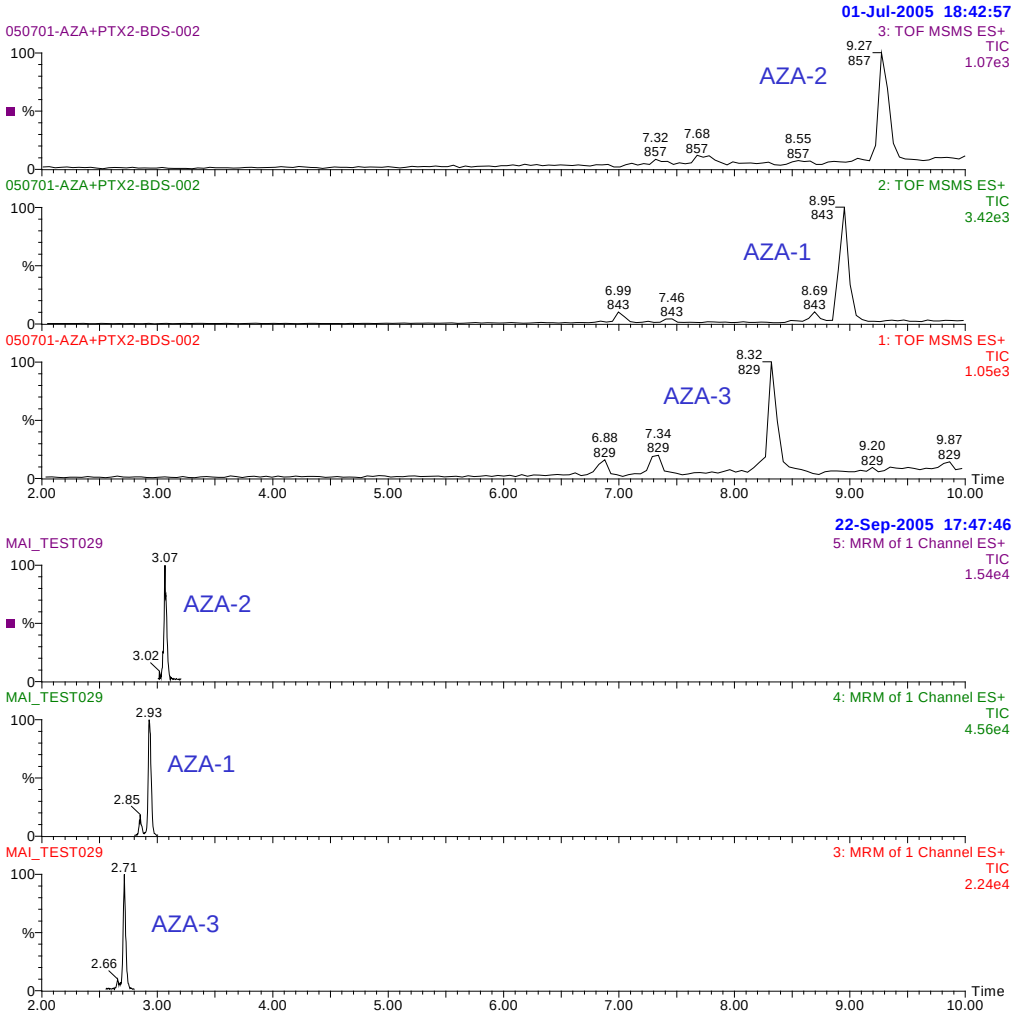
Conditions:

Acquity C8 (50×2mm, 1.7µm)

2mM ammonium formate, 50mM formic acid

Gradient conditions

Flow rate 0.4mL/min



Fux E., McMillan D., Bire R., Hess P., (2007) Development of an Ultra Performance Liquid Chromatography – Mass Spectrometry method for the detection of lipophilic marine toxins. J. Chromatogr. A 1157, 273-280.

→ **Highly cited paper award (cited 35 times in 3 years) !!**

Analysis of the FDMT-material by LC-MS/MS

Conditions:

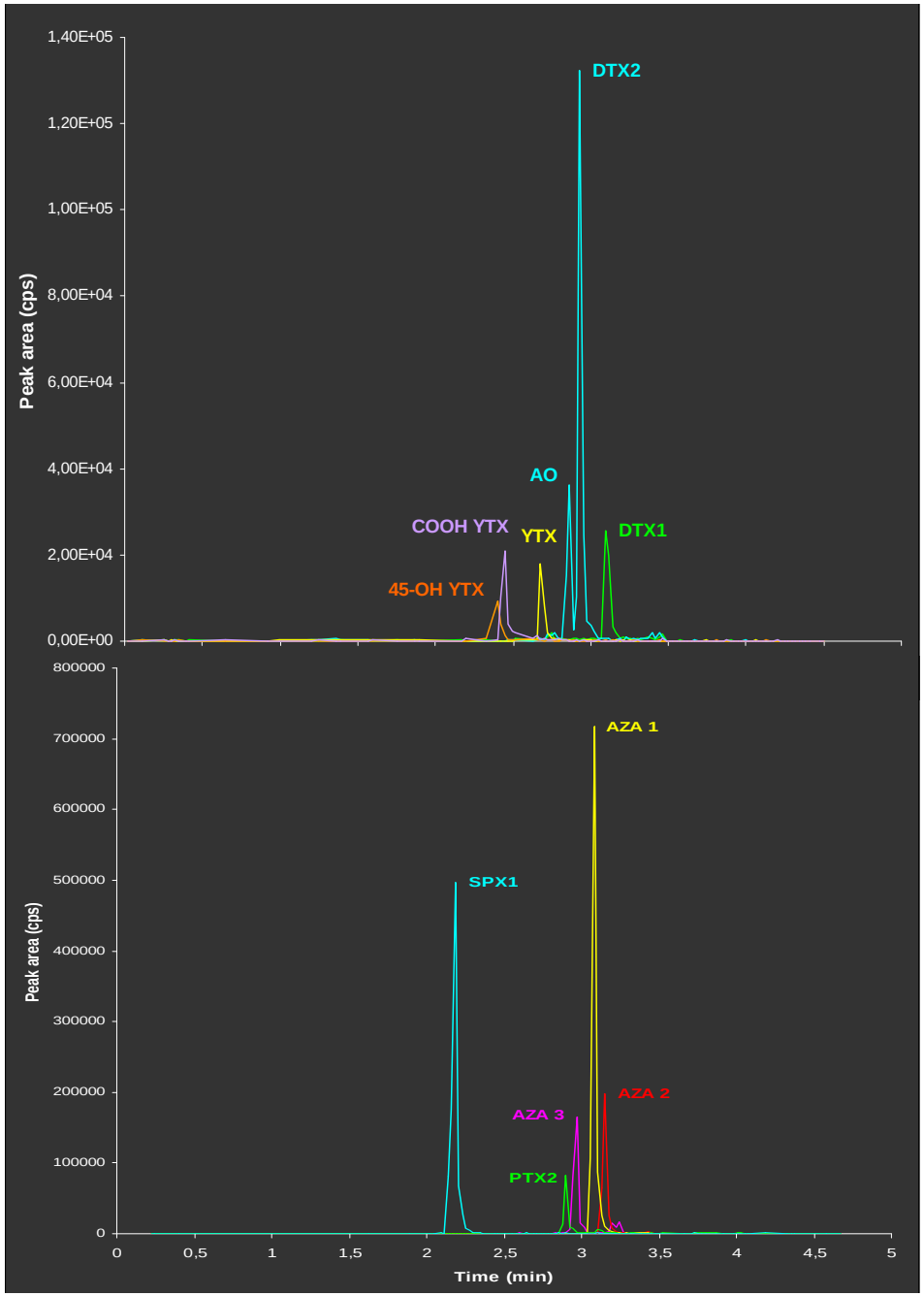
API5500 Q-Trap

Kinetex C18 (50×2mm, 2.7 μm)

2mM ammonium formate, 50mM formic acid , temperature: 40°C

Gradient, flow rate 0.5mL/min

Ifremer, 2010
Unpublished data



Influence of sample strength on matrix effect in LC-MS

BIOTOX (EU FP7)

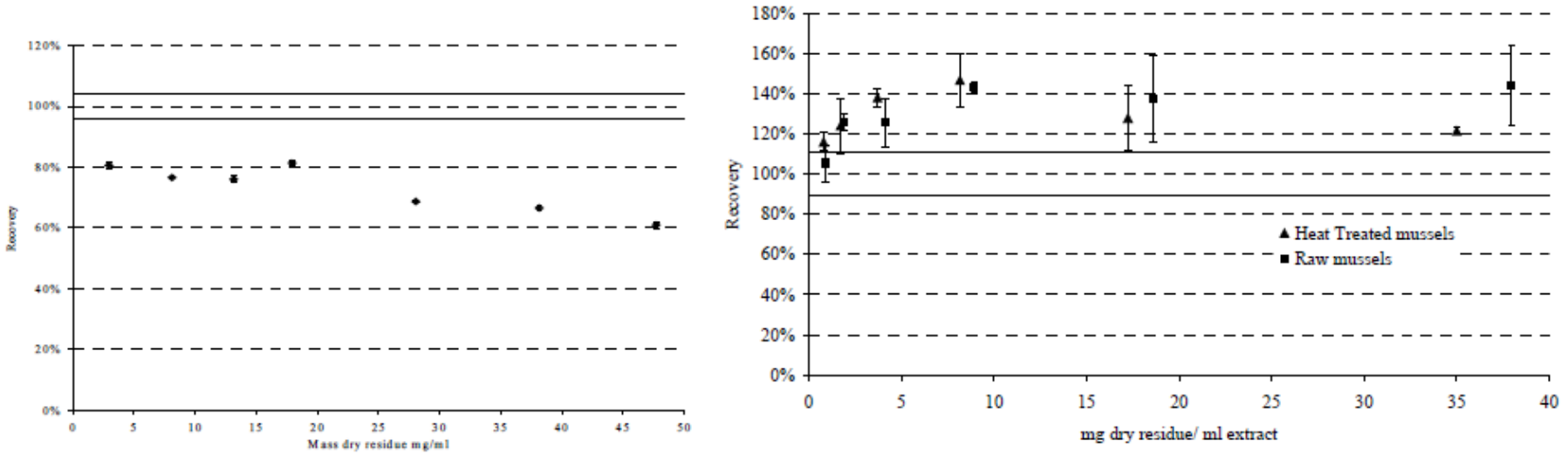
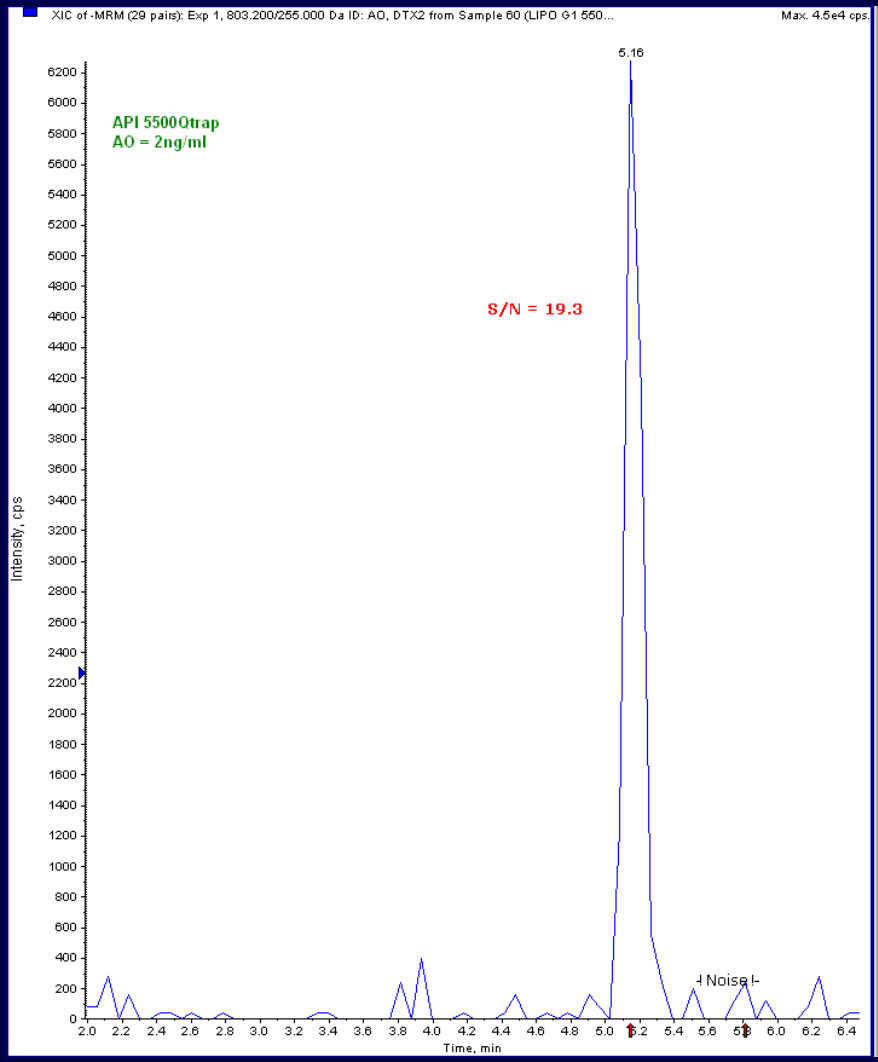


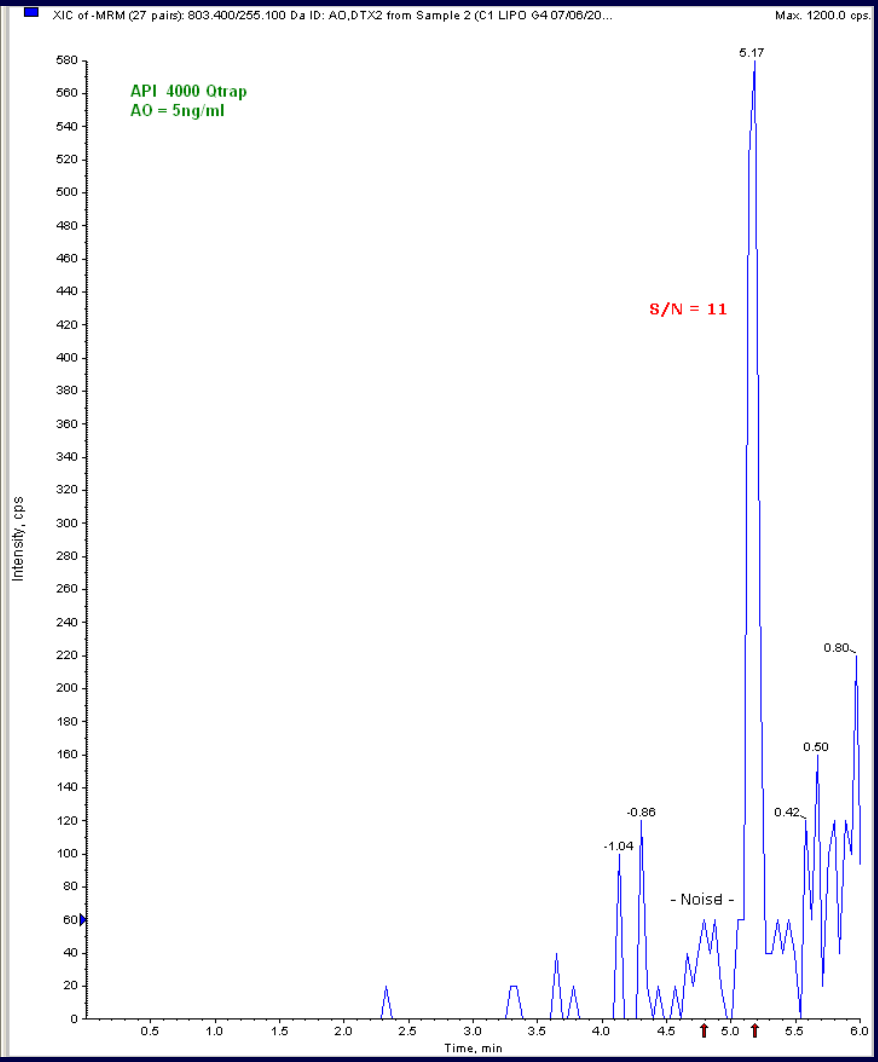
Figure 2.2.9. Post extraction addition of AZA (left graph) and OA (right graph) analysed using step gradient HPLC conditions and Q-Tof detection. Bold lines represent the precision obtained

Fux E., Rode D., Bire R., Hess P. (2008) Food Addit. Contam. 25 (8), 1024-1032.

→Large dilution necessary to overcome matrix effects in crude extracts (< 1 mg sample / mL extract) !!

Comparison of sensitivity : API4000 and API5500

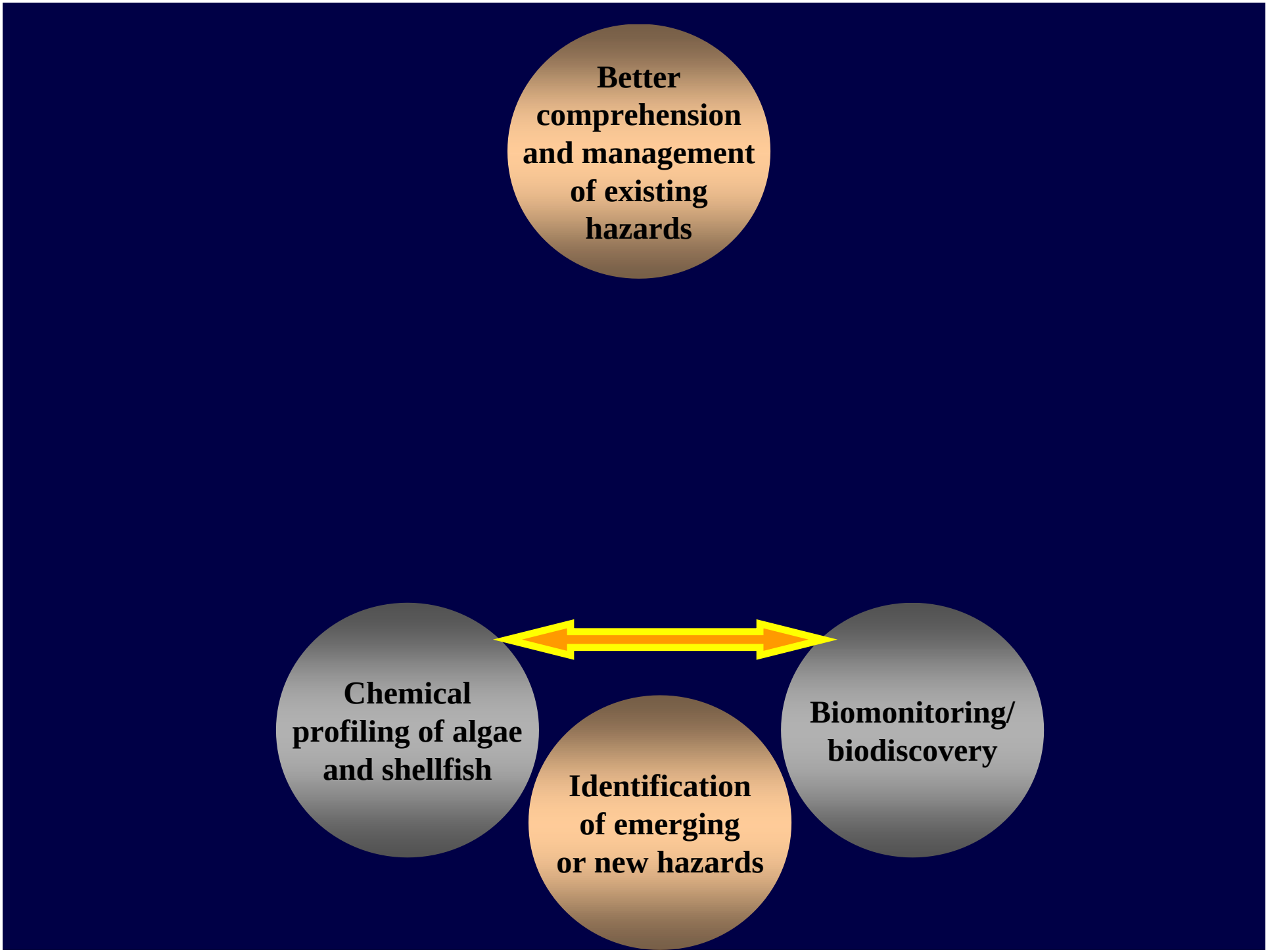




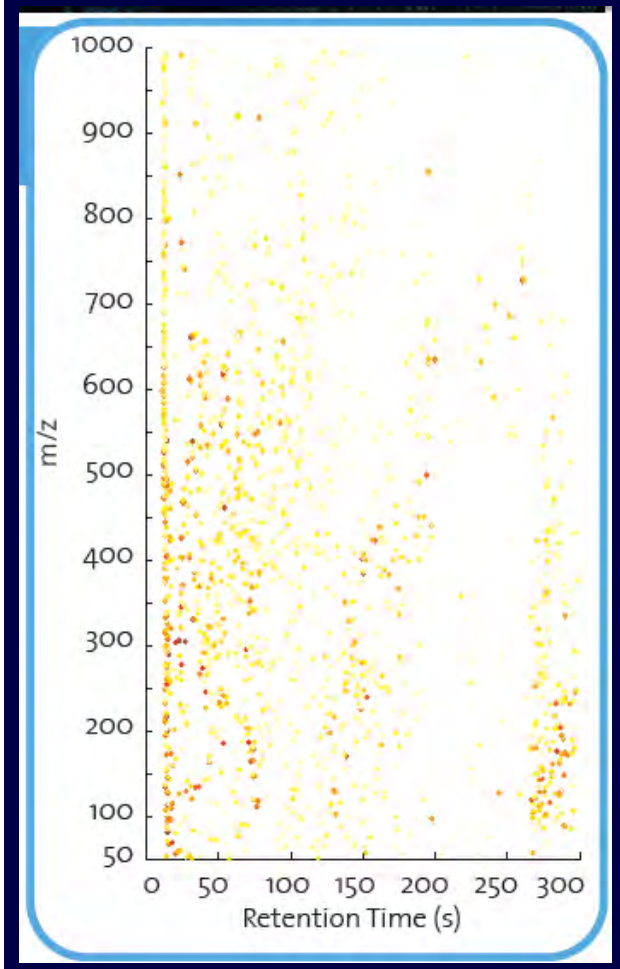
→ Gain of factor 5 ! → Dilution is the solution !!

Table 1: Precision data for AZA1

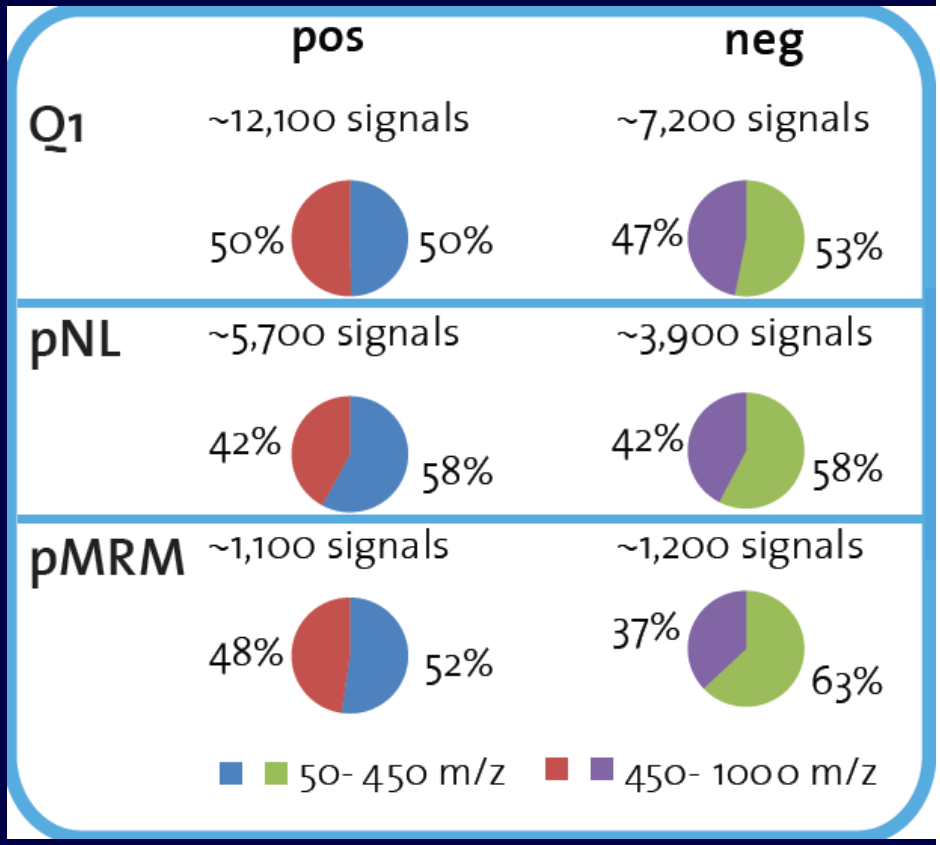
	Mean value (in µg/kg)	Repeatability conditions	Intermediate conditions	Reproducibility conditions	HORRAT (uncorr.)	HORRAT (corr.)
Samples 1/3: Blue mussels, cooked **Laboratories after outlier elimination [Outliers]: 13 [0]**						
Homogenate	96.36	11.8%	14.1%	21.2%	0.93	0.96
Sample 5 (Extract of samples 1/3) **Laboratories after outlier elimination [Outliers]: 11 [2]**						
Extract	111.74	8.0%	9.6%	23.2%	1.04	1.05
Samples 6/8: Oysters, raw **Laboratories after outlier elimination [Outliers]: 13 [0]**						
Extract	99.46	10.5%	11.9%	22.6%	1.00	1.03
Sample 4: Blue mussels, cooked						
Homogenate	< LOD					
Sample 7: Clams, raw **Laboratories after outlier elimination [Outliers]: 12 [1]**						
Extract	82.50	9.9%	10.7%	14.7%	0.63	0.67
Samples 9/11: Blue mussels, cooked **Laboratories after outlier elimination [Outliers]: 7 [0]**						
Extract		5.1%				
Homogenate	17.42	10.4%		37.9%	1.29	1.72
Samples 10/12: Blue mussels, cooked **Laboratories after outlier elimination [Outliers]: 7 [0]**						
Extract		3.6%				
Homogenate	71.86	4.9%		30.8%	1.30	1.40
Sample 13: Blue mussels, raw **Laboratories after outlier elimination [Outliers]: 15 [0]**						
Homogenate	127.54	9.7%		15.2%	0.69	0.69
Sample 14: Blue mussels, raw **Laboratories after outlier elimination [Outliers]: 15 [0]**						
Homogenate	64.92	7.5%		16.3%	0.67	0.74
Sample 15: Blue mussels, raw						


Better comprehension and management of existing hazards
Chemical profiling of algae and shellfish
Identification of emerging or new hazards
Biomonitoring/ biodiscovery

Untargeted profiling
example of yeast metabolome
HRLC – scanning on MS API 5500



5 min



Translation of 1000s of signals into 100s of compounds !

COLNACOQ - Bioactivity of fungal and microalgal metabolites in the environment surrounding shellfish

Thèse Marie Geiger: Development of a bioassay suite for bioguided fractionation

Supervisory team: YF. Pouchus, O. Grovel, J. Boustie, V. Séchet, J. Dupont

2 Objectives:

- To develop and validate a series of bio-assays for the discovery of lipophilic bio-active compounds in the marine environment

- To apply these bio-assays to the screening of various marine microorganisms (focus on micro-algae and micromycetes) and contaminated shellfish

- 3 types tests -

- Antibacterial activity
- Cytotoxicity
- Insect larval toxicity

Discovery of antibacterial activity in P. expansum

Marie Geiger, 2010

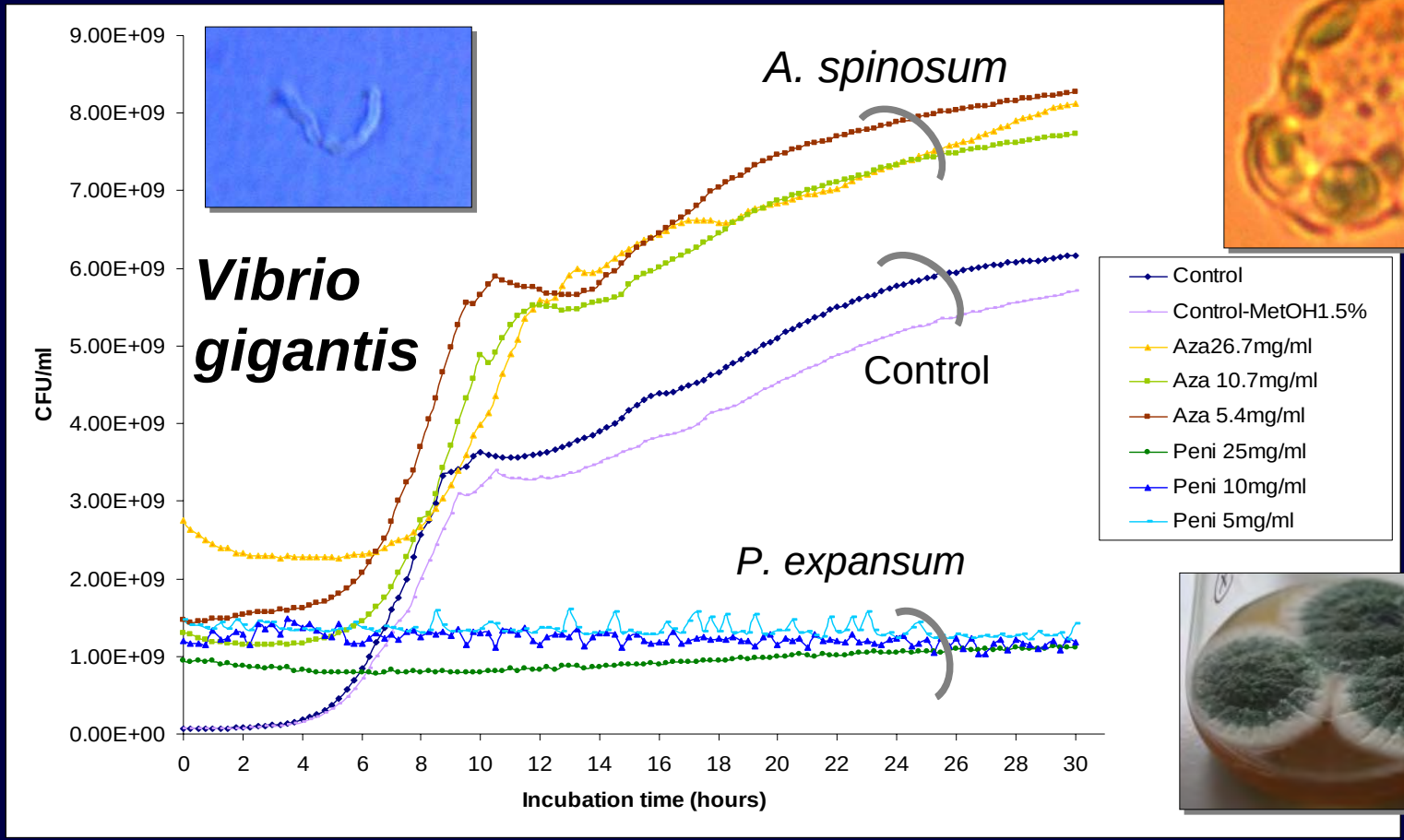
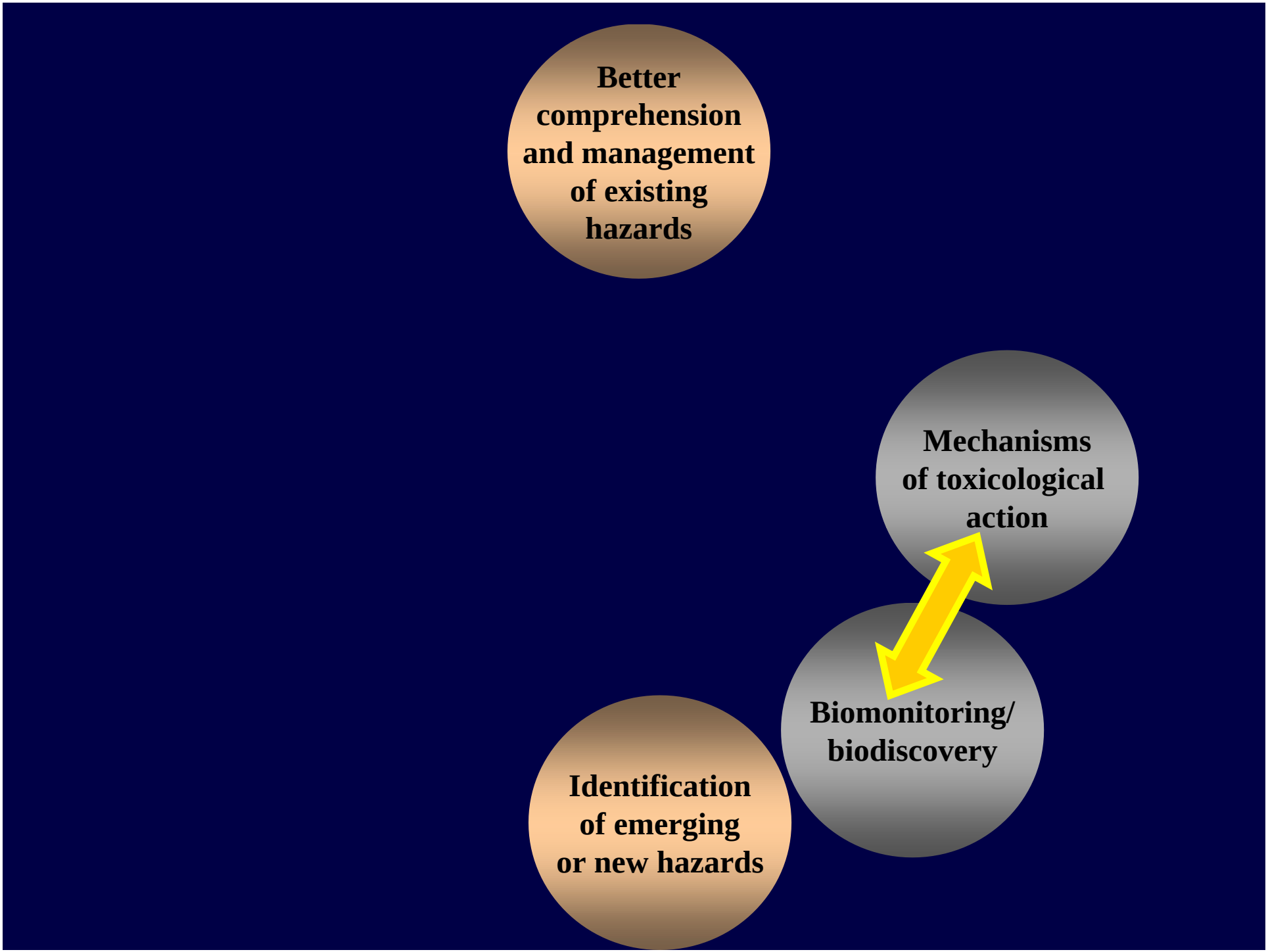
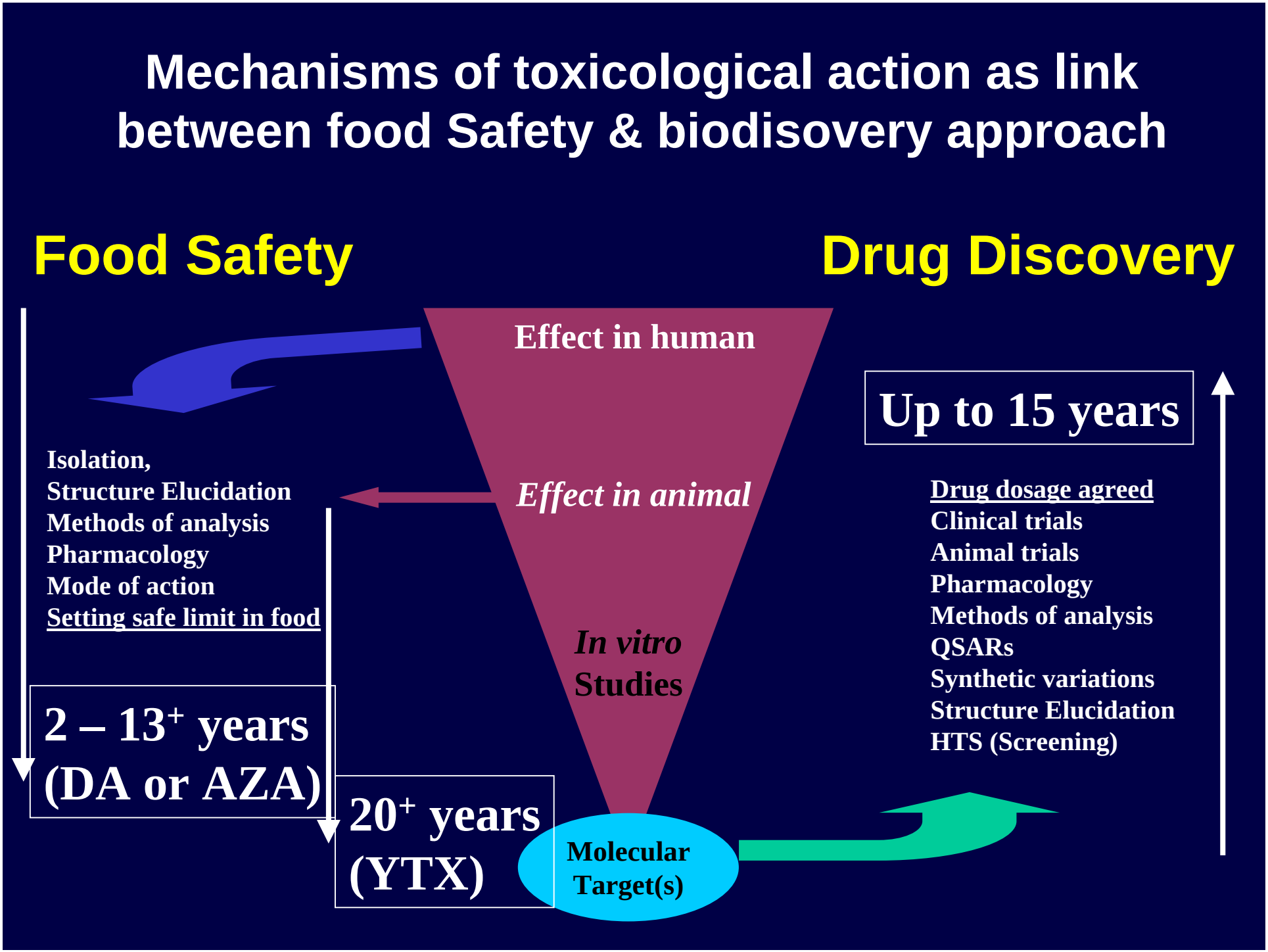
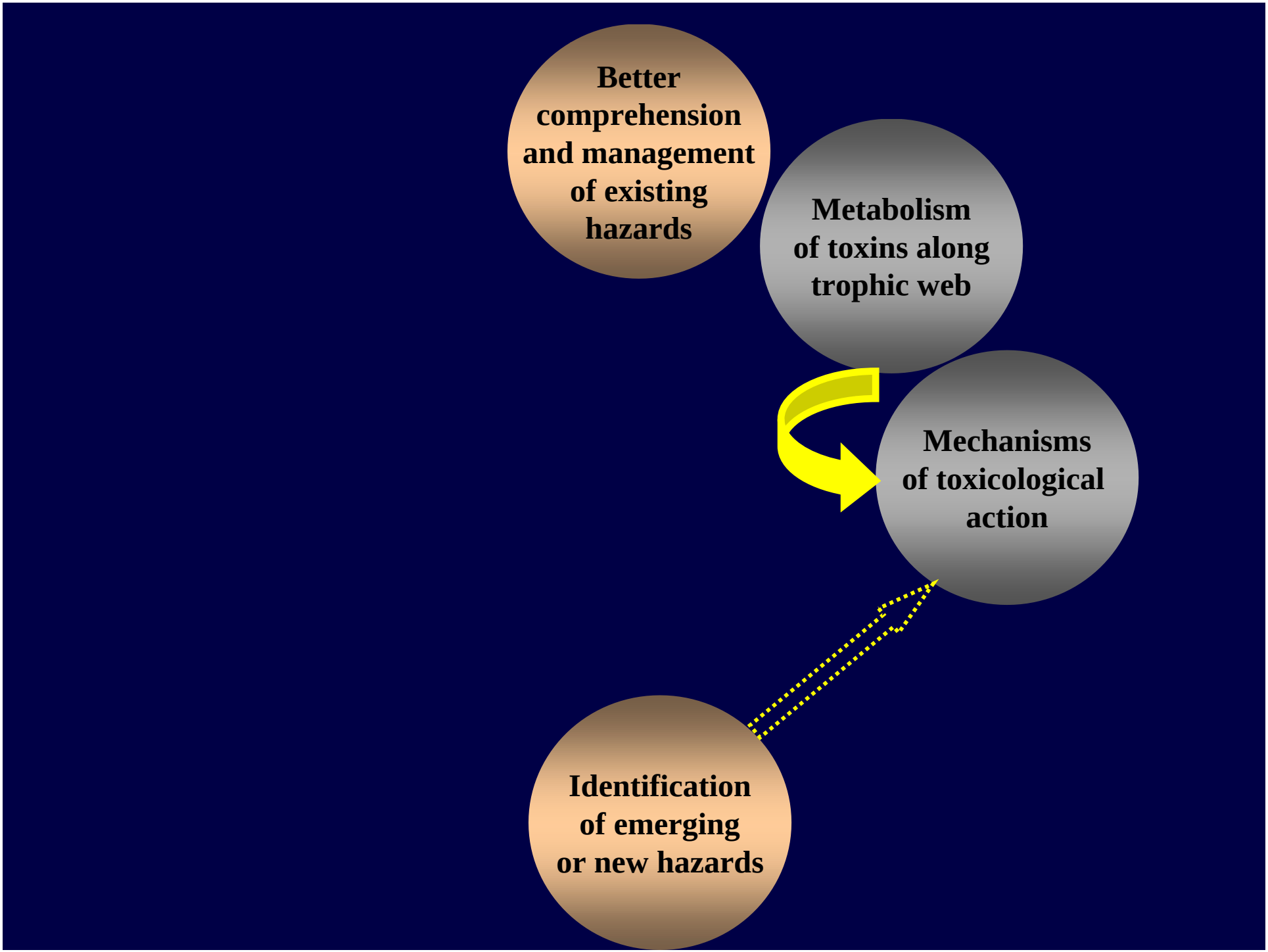


Figure 6: Preliminary antibacterial assay with crude extracts of *A. spinosum* and *P. expansum* tested on *V. crassostreae* (concentrations are given in mg dry residue crude extract per mL of final well volume)


Better comprehension and management of existing hazards
Mechanisms of toxicological action
Biomonitoring/ biodiscovery
Identification of emerging or new hazards

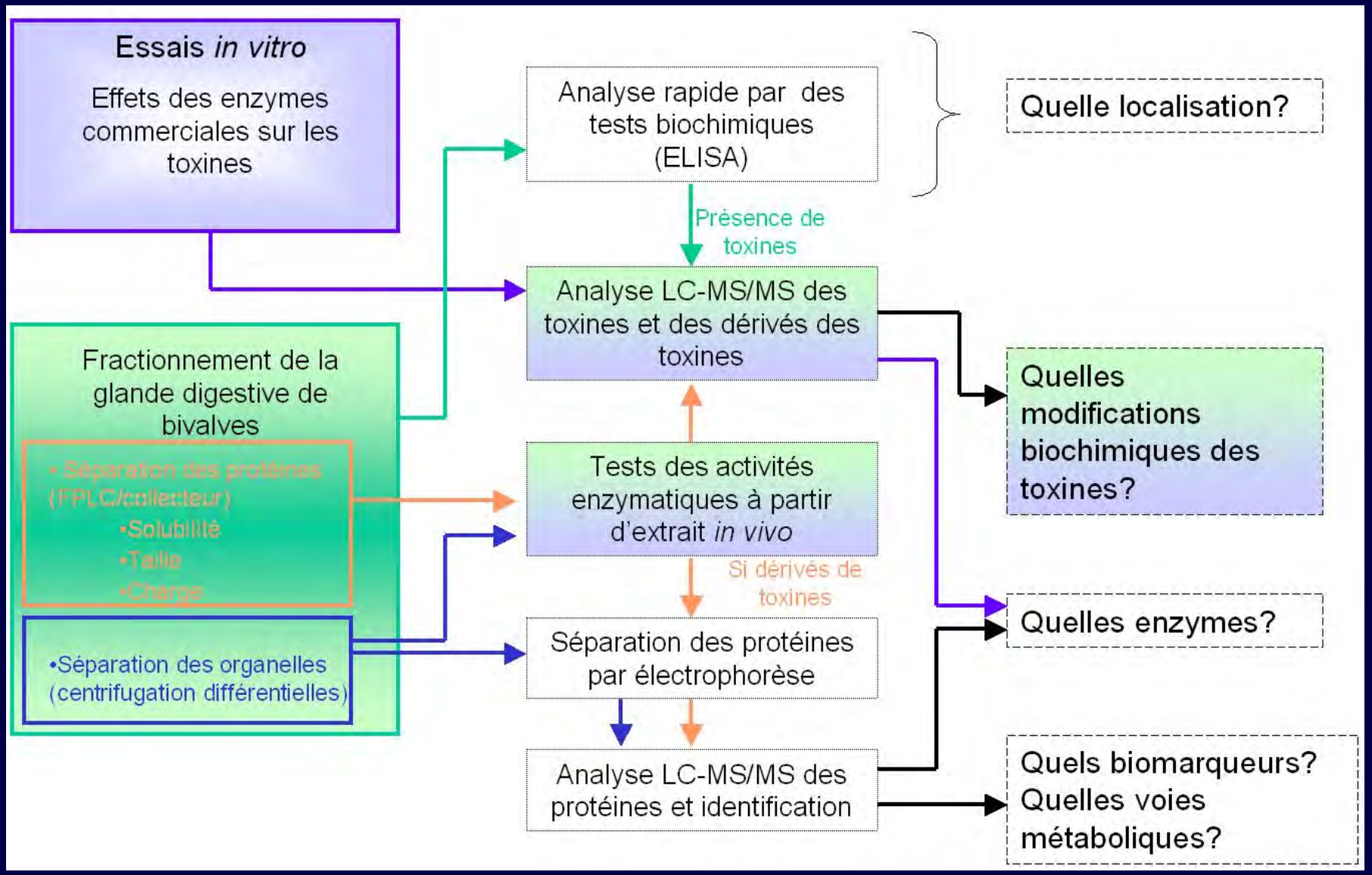
Mechanisms of toxicological action as link between food Safety & biodisovery approach

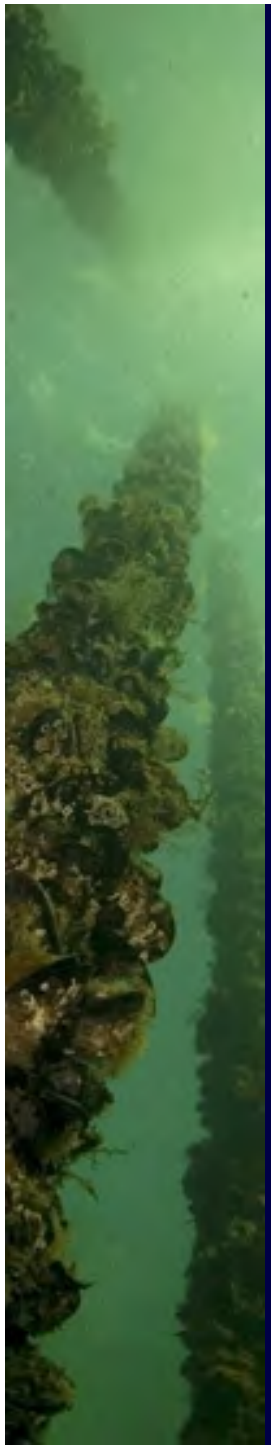



Better comprehension and management of existing hazards
Metabolism of toxins along trophic web
Mechanisms of toxicological action
Identification of emerging or new hazards

Toxin – protein interactions

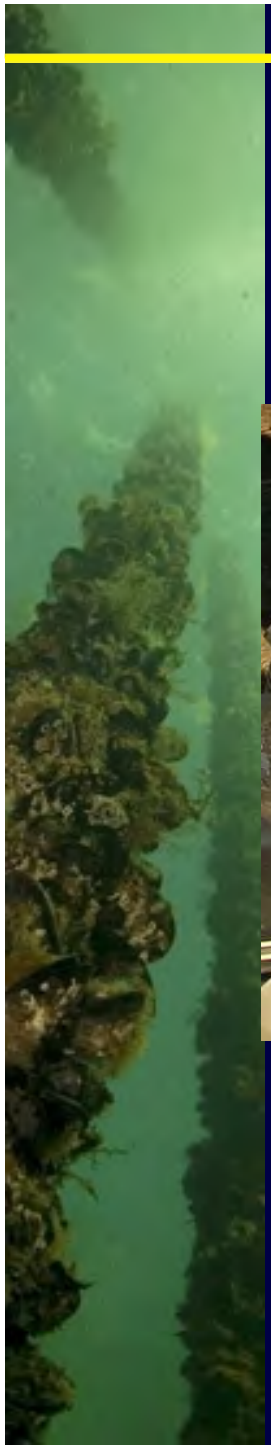
Elodie Nicolau, 2010





Summary - 2

- ✓ Networking efforts combined with a systematic approach allow for multiple studies, as long as funding is available and priorities can be agreed upon.
- ✓ Recent legislative developments and industry requirements require further efforts in the following areas:
  - ✓ Production of purified toxins (to calibrate & establish TEFS)
  - ✓ Production of RMs & method validation
  - ✓ Biological surveillance techniques: High-throughput screening of multiple activities
  - ✓ Better understanding of shellfish metabolism and mechanism of action of toxins in mammals
  - ✓ More efficient detection of new or emerging toxins (HTS HRLC – HRMS techniques)
  - ✓ More efficient monitoring of algae (satellite, flow cytometry, probes, taxonomy)



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Merci pour votre attention !!