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SMR 1550 - 3

WORKSHOP ON THE USE OF RECEPTOR BINDING ASSAY (RBA)

1 - 5 September 2003

Co-organized by the International Atomic Energy Agency (I.A.E.A.)

New Methods for Analysis of Marine Biotoxins

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These are preliminary lecture notes, intended only for distribution to participants.

New methods for analysis of marine biotoxins

Requirements for validation and regulatory acceptance

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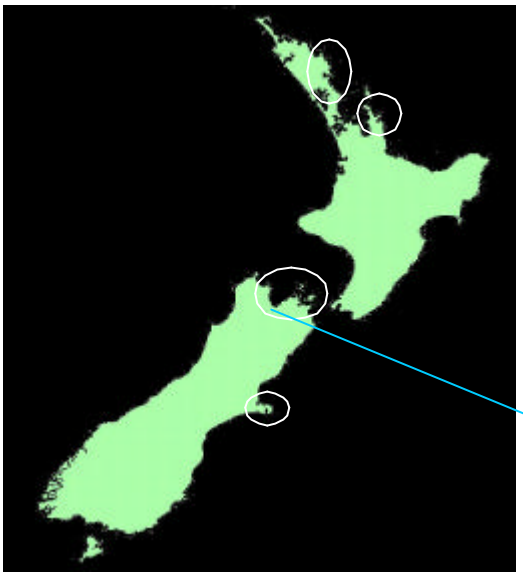
NEW ZEALAND

New Methods for Marine biotoxins

- A new NZ programme
- New test methods
- LC-MS/MS method - ASP/DSP toxins
- Validation
- Example - Akaroa
- Issues & Summary

New Zealand Aquaculture



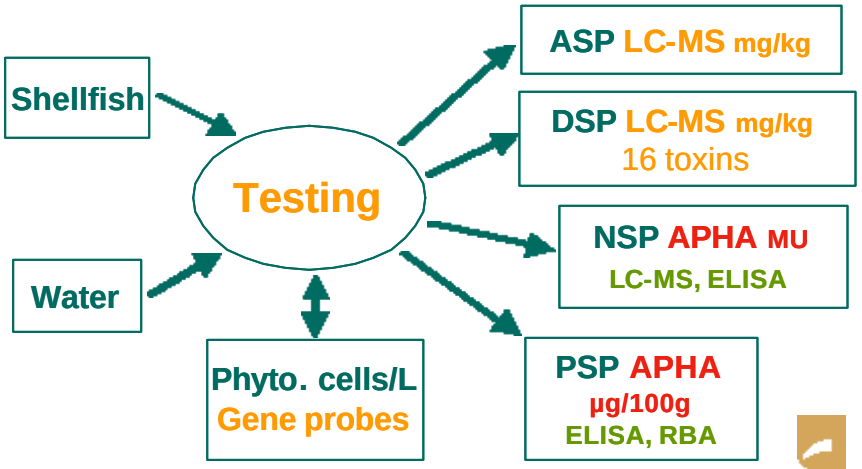
- NZ$300 million
- Greenshell™ mussel
- Scallop
- Pacific oyster
- Clam & cockle
- Abalone

Aquaculture - Marlborough Sounds

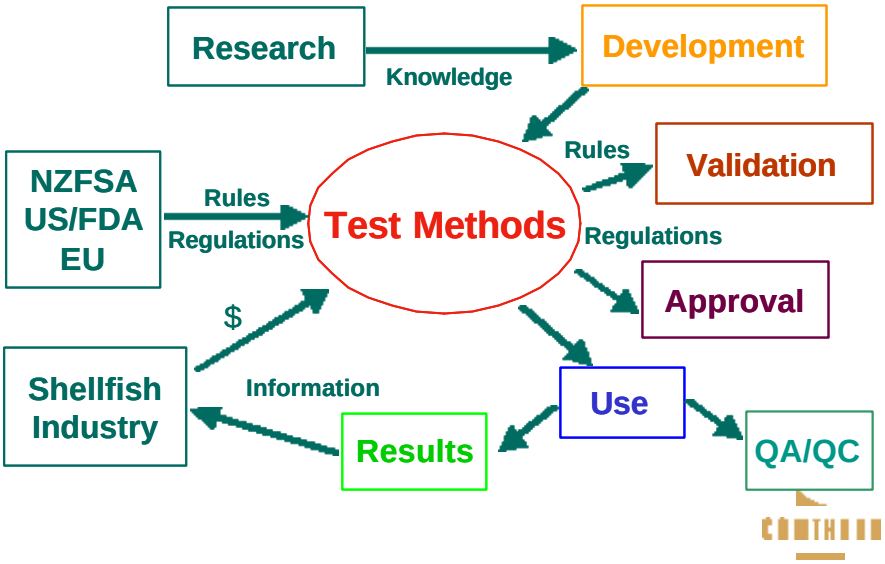
Marine biotoxin management in NZ

- **Shellfish industry programme**
  - export oriented
  - locally managed, 100% industry funded
  - regulated by NZ Food Standards Authority
  - audited by FDA and EC
- **Public health programme**
  - recreational and customary use
  - managed and funded by NZFSA

The new NZ programme

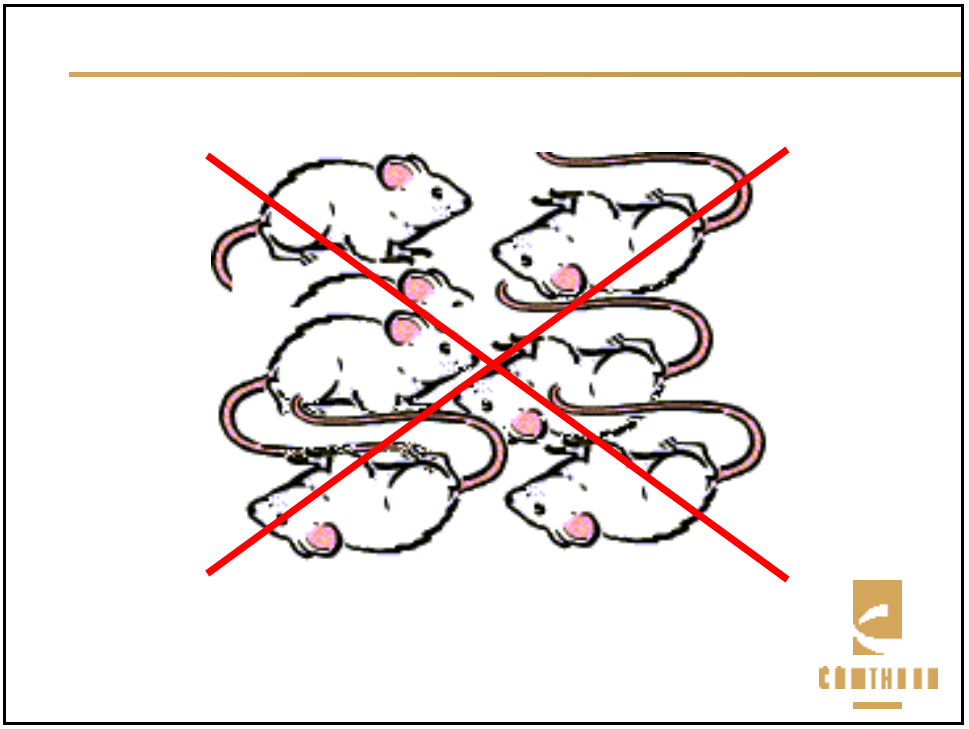


New processes



Why new test methods? – the 4 S's

Speed	Results in hours, not days
Sensitivity	Early warning; tracking; no false negatives
Specificity	No false positives; i.d. of toxins
Sustainability	Ethical testing, no small animals



N.Z. requirements for new methods

- Reliable to detect and quantify biotoxins
- Cost effective
- Validated and assessed - "fit for purpose"
- Internationally accepted (market access)

Assay Technologies

Receptor Radio-ligand binding; Cyto-toxicity; Fluorescent ligand binding, etc.

Antibody ELISA plate; Strip (Rapid-ALERT)

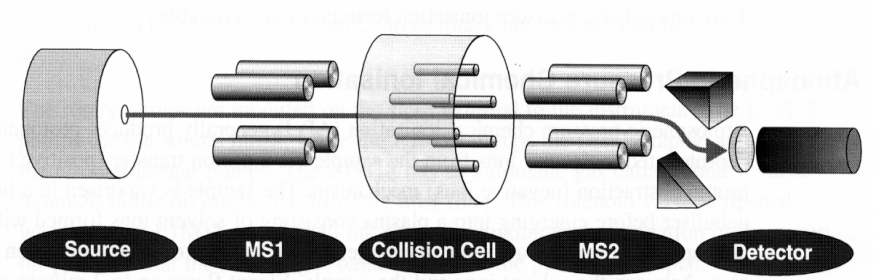
Enzyme Protein-phosphatase; Cholinesterase

Chromatographic
LC-UV, LC-FL, LC-MS, LC-MS/MS

LC-MS/MS method for ASP & DSP toxins

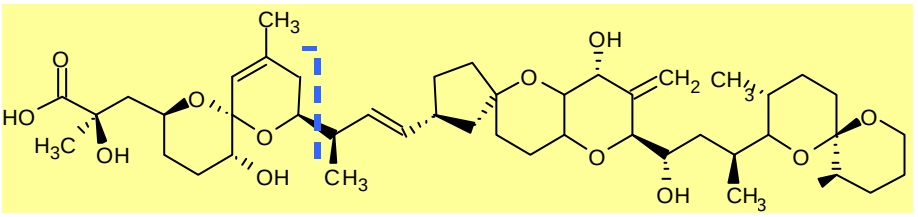
- Extraction of shellfish homogenate:
  2g + 18ml 90% methanol
- Hexane wash
- LC-MS: 2 x 150mm C18 column
  0.2 ml/min ACN gradient + buffer
  (46mM formic acid + 4mM NH_4Acetate)
  ESI + and -, MRM 13 channels
- Toxins detected - 17 toxins in 6 classes
- Automated injection and data processing (ca 30 shellfish samples per day)

Mass spectrometry (MS/MS)



ionisation select parents fragment select daughters detect

LC-MS/MS of Okadaic acid



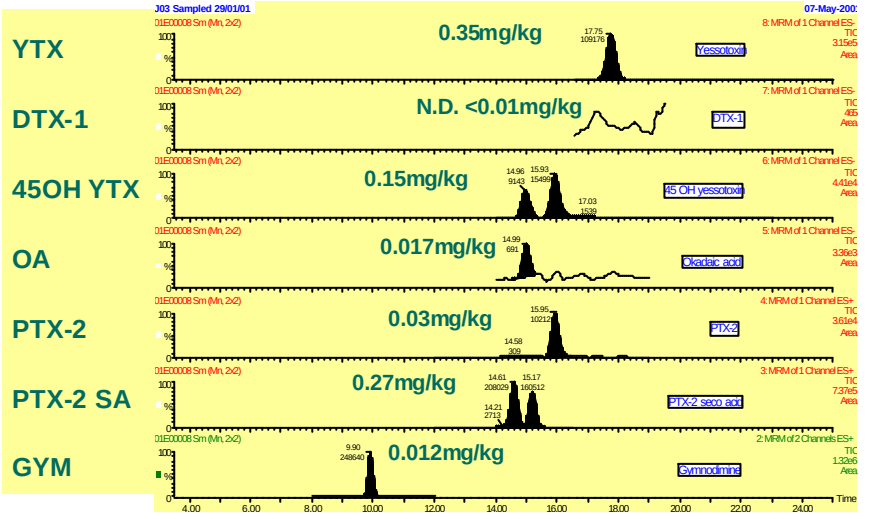
803 $[M-H]^-$

255 loss of 548

ASP & DSP Toxins by LC-MS/MS

Domoic acid	DA	Quantitative
Okadiac acid	OA	Quantitative
Pectenotoxin-2	PTX2	Quantitative
Yessotoxin	YTX	Quantitative
Gymnodimine	GYM	Quantitative
OA esters (by hydrolysis)	DTX3	Quantitative
DTX1, DTX2		Semi-quantitative using RFs
PTX1, PTX6, PTX-seco acids		
45OH-YTX, homo-YTX, carboxy-YTX		
Azaspiracid-1, -2, -3	AZAs	
Spirolides	SPXs	

Contaminated Mussel Sample



Method Validation - Within-lab

- N.Z. procedure described : *www.maf.govt.nz/standards/seafood/guidelines*
- Performance based
- Follows international protocols to fully characterize a method:
  CODEX, AOAC, IUPAC, Eurachem

CODEX Method Classification

Type I – Empirical method
Results and limits defined by the method
e.g. mouse bioassays

Type II – Reference method
Key method for discrete substance(s)
e.g. ASP toxins by HPLC-UVD

Type III – Alternate method
Also approved as meeting validation criteria

Type IV – Candidate method

CODEX Type II & III Methods

- Accuracy / recovery
- Applicability – matrices, range
- LOD and LOQ
- Precision – within-lab; inter-lab.
- Selectivity
- Linearity
- Practicality

ASP/DSP Method Validation

- Protocol 500 LC-MS runs, 1200 hours
  - recoveries - 4 shellfish species x 2 levels
  - extractability
  - mouse bioassay comparisons
  - hydrolysis of OA/DTX1esters
  - uncertainty
- Approvals - ISO 17025
  - NZFSA
- Inter-laboratory study completed (8 labs)

ASP/DSP Method: within-lab parameters

Limit of Detection	0.001 – 0.01 mg/kg	DA 0.02 mg/kg
Working Range	LoD – 2 mg/kg	
Precision - RSD_R	10 – 25% at 0.05 – 0.1 mg/kg	8 - 13% at 0.5 – 2 mg/kg
Accuracy	Bias < 10% for DA, OA and DTX-1	NRC CRMs

Inter-laboratory Studies

- Method precision under reproducibility conditions, RSD_R
- Comparison to analytical norms
  - AOAC : Horwitz bell curve
  - CODEX : RSD_R < 23% at < 0.18 mg/kg.
- Uncover operational problems
  - labs
  - instruments

Inter-lab Study of ASP/DSP Method

- Coordinated by Cawthron Institute, 2002
- 10 labs - 8 labs returned triplicate data:
  Australia, Canada (2), Ireland, Japan, Netherlands, Norway, NZ.
- 5 standard solutions 5 – 200ng/mL :
  AZA1, DA, Gym, OA, PTX2, YTX
- 4 mussel extracts, toxins 0.03 – 3 mg/kg
- Precision analysis of data per ISO 5725 guide for inter-lab studies

Inter-lab Study - Results

- Excellent calibrations R^2 >0.98
- Repeatability 8-12%, except PTX2
- Reproducibility:

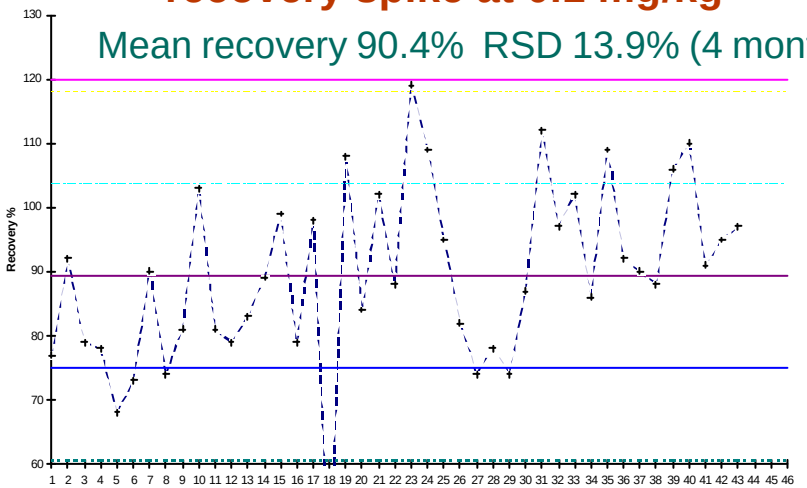
Toxin	AZA1	DA	Gym	OA	DTX2	PTX2	YTX
mg/kg	0.42	1.67	0.06	0.04 - 0.28	1.59	0.04 - 0.11	1.7 - 2.9
RSD_R	34%	23%	42%	25% - 17%	12%	54% - 44%	22%- 15%
Horrat	1.9	1.6	1.7	0.9	0.8	1.9	1.3

QA/QC – Performance Verification

- Calibration linearity
- Duplicate samples
- QC sample – all analytes
- Blank sample
- Recovery – fortified blank

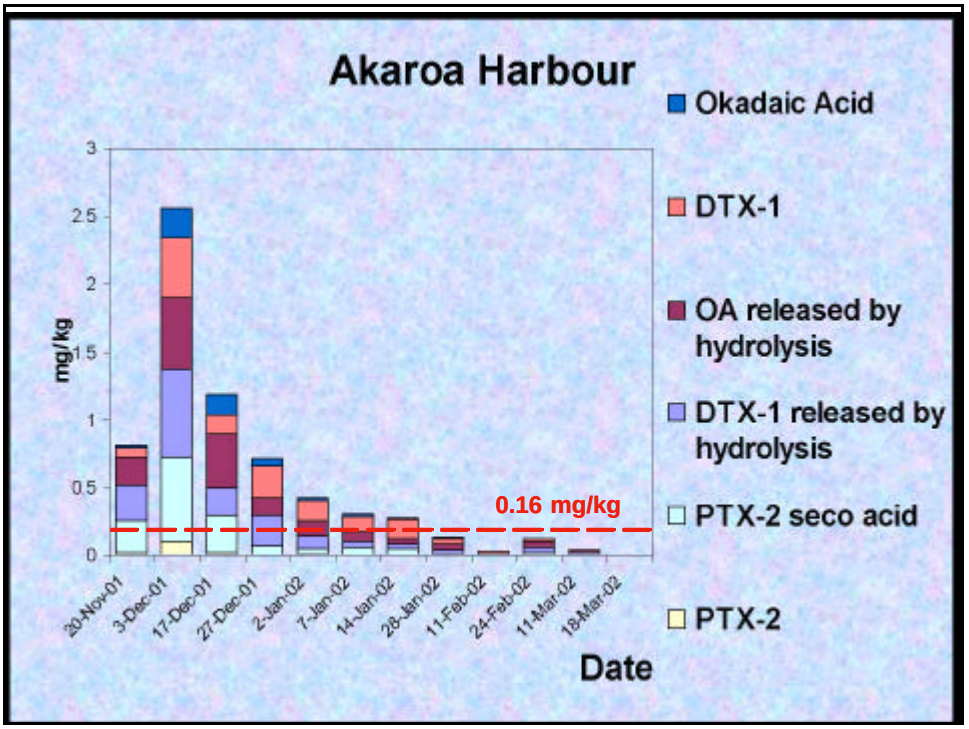
ASP/DSP Control Chart – okadaic acid recovery spike at 0.1 mg/kg

Mean recovery 90.4% RSD 13.9% (4 months)



Akaroa Harbour DSP incident

- Phytoplankton bloom - *Dinophysis acuminata*
- Nov. 2001 – Jan. 2002
- Shellfish from 3 sampling sites - weekly
- LC-MS testing
- Public health protected



Issues

- Analytical standards and CRM's
- Metabolites and toxicology
- Regulatory limits for each toxin class
- Pathway to international acceptance?
  - CODEX, AOAC, EU/CEN
- Training: lab staff, regulators, clients
  - methods and results more complex

Summary

- Full method validation is complex but essential for acceptance of new methods
- ASP/DSP method has realized benefits :
  - fast turnaround with precise results,
  - protected public health
  - cost reduction,
  - no use of animals,
  - more knowledge on marine biotoxins
- Need for better screening assays for PSP and NSP

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Tracey Neil, Andy Selwood, Karyn Bell
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HABTech 2003

26-30 Nov., Nelson, N.Z.

Workshop on technologies for monitoring of HABs & marine biotoxins

- Plenary lectures
- Hands-on demonstrations (incl. LC-MS, ELISA, RBAs)
- Field trip
- See web page:

http://www.cawthron.org.nz/habtech03

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