
# Official control monitoring of marine shellfish in UK waters: methods and approaches

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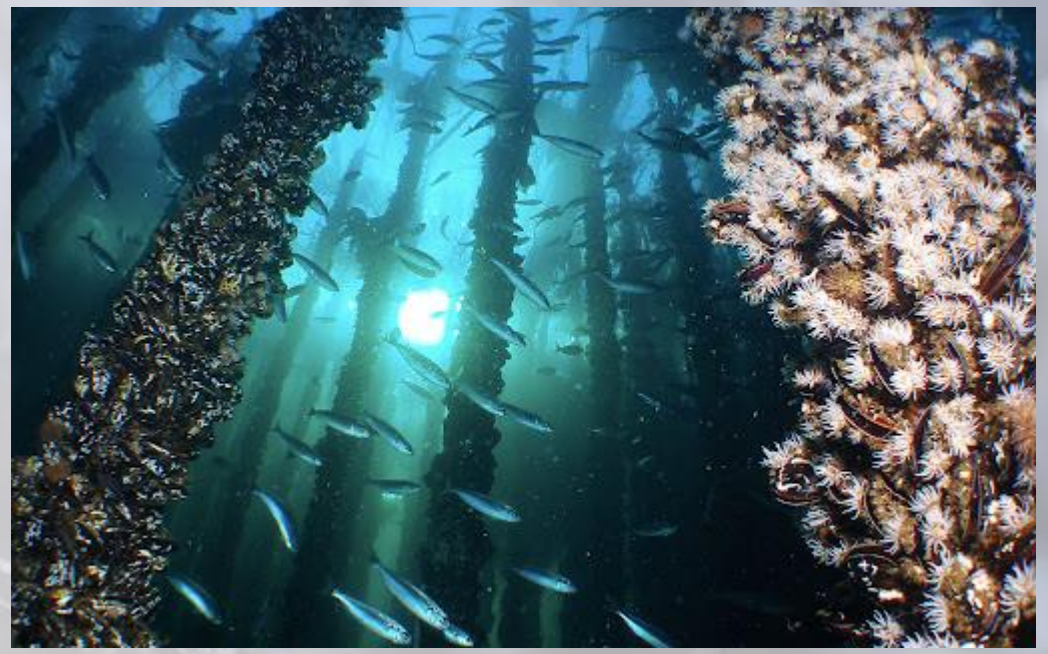
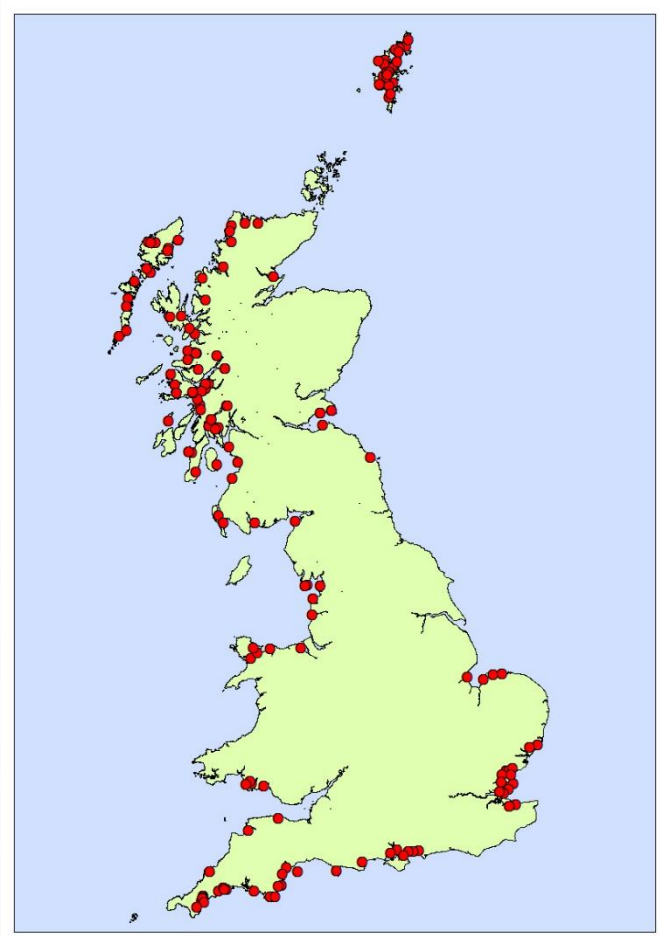
# Official control monitoring of marine shellfish in UK waters: methods and approaches

- **Overview**

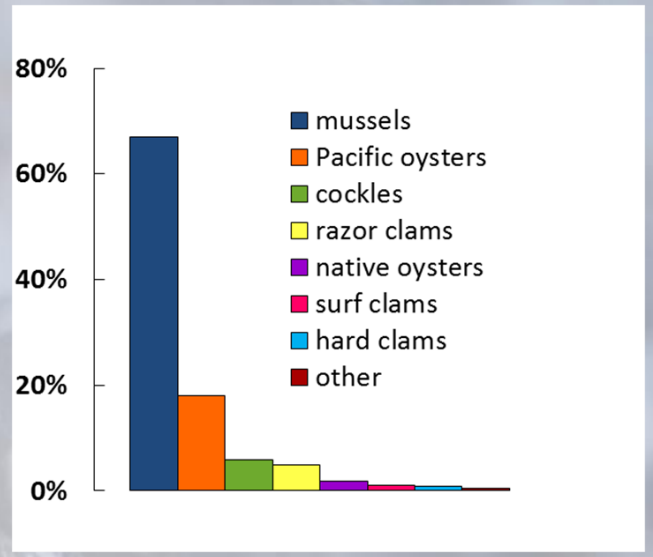
- Official Control Methods used in EU/UK
- Phytoplankton sampling and analysis
- Marine toxin sampling and analysis
- Chemical contaminants and methods



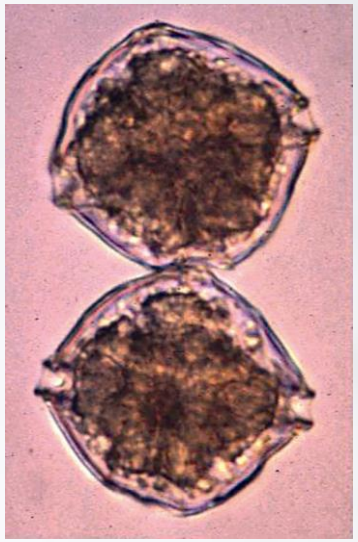
# Official Control Monitoring



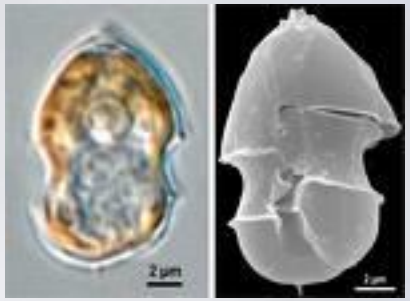
- HAB phytoplankton and biotoxin monitoring programme
  - Samples of are collected from pre-determined monitoring points (weekly-monthly)
  - ~ 170 monitoring points
  - Toxins results reported 1 day after receipt
  - Phyto 2 days after receipt
- Chemical contaminant monitoring
  - Annual, pre-selected points
  - Reported within 1 month



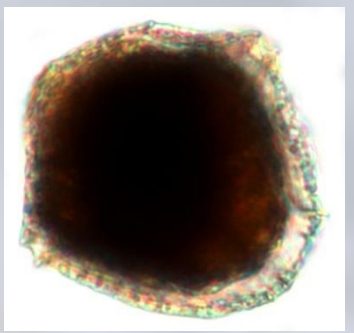

# Toxin-producing species



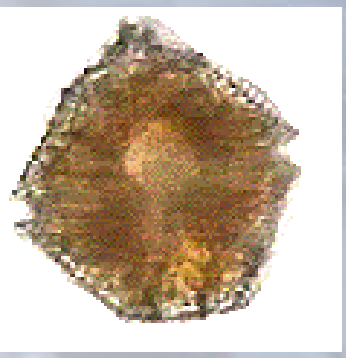
*Alexandrium sp.*



*Azadinium spinosum*



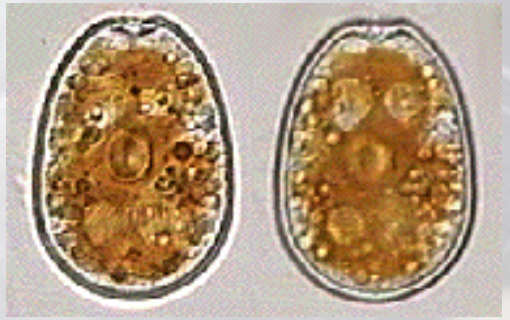
*Protoceratium reticulatum*



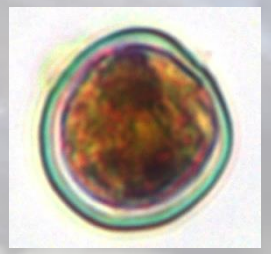
*Lingulodinium polyedrum*

*Pseudonitzschia sp.*

*Dinophysis sp.*



*Prorocentrum lima*



*Prorocentrum minimum*

# Purpose & benefits of water monitoring

- **Requirement of legislation**
- **Early warning system**
  - **Safeguard public health**
  - **Minimise losses to industry**
- **Better understanding of temporal and spatial distribution of toxin-producing species**

# Sample Collection

Samples must be **representative** of the algal community in the water body being sampled.

- Ideally, samples should be taken from over the shellfish beds at high water (+/- 1hr)
- Various sampling methods
  - Tube / pole samplers
  - Nets
  - Surface water
- Cells are easily damaged – sample must be fixed as soon as possible after collection to keep cell integrity.
- Equipment must be rinsed prior to and after collection.

# Phytoplankton testing

- Representative samples collected
  - Fixed with Lugol's iodine
- Water samples arrive ~9:00 am (Tue-Fri)
- Homogenisation (mixing)
- Dispense into Utermöhl chambers
  - Leave for 24hours to settle

- Analysis by base plate count of HAB taxa

# Toxin Testing

# Toxin testing

- **Representative** samples collected

- Shellfish bags arrive ~7.30 am (Tue-Fri)
- Homogenisation (blending)
- Separate into three tests (ASP, LT, PSP)

*All tests involve*

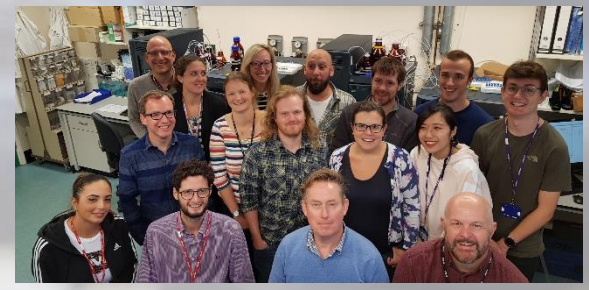
- Solvent extraction (to remove toxins from shellfish)
- Clean-up (chemical and/or physical)
- Analysis
  - Separation
  - Detection











# Shellfish testing process

- Samples received - daily
- Shellfish shucked, >50g tissue homogenised
  - Min 10 organisms per sample
- Sub-samples for each of three testing methods
- Extraction, clean-up
- Analysis overnight
- Results reported next day (customer requirement)

# What happens if toxins/harmful plankton is detected ?

## Shellfish flesh – EU regulatory limits

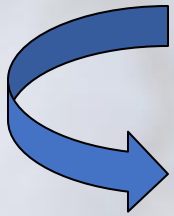
PSP: 800 µg/kg flesh

ASP: 20 mg/kg flesh

OA/DTX/PTX: 160 µg/kg flesh

AZA: 160 µg/kg flesh

YTX: 3.75 mg/kg flesh



If regulatory limit exceeded

close area/recall product

Continue monitoring -- 2 negative results allow reopening of area

## Water - Trigger levels (UK example)

PSP producing algae: Presence (40 cells/L)

ASP prod. algae: 150,000 cells/L

DSP prod. algae: 100 cells/L

If trigger levels exceeded

Increase monitoring

# Current testing capabilities

## *Toxin methods*:

- ASP – LC-UV
- PSP – LC-FLD
- LTs – LC-MS/MS

Methods written in European regulations







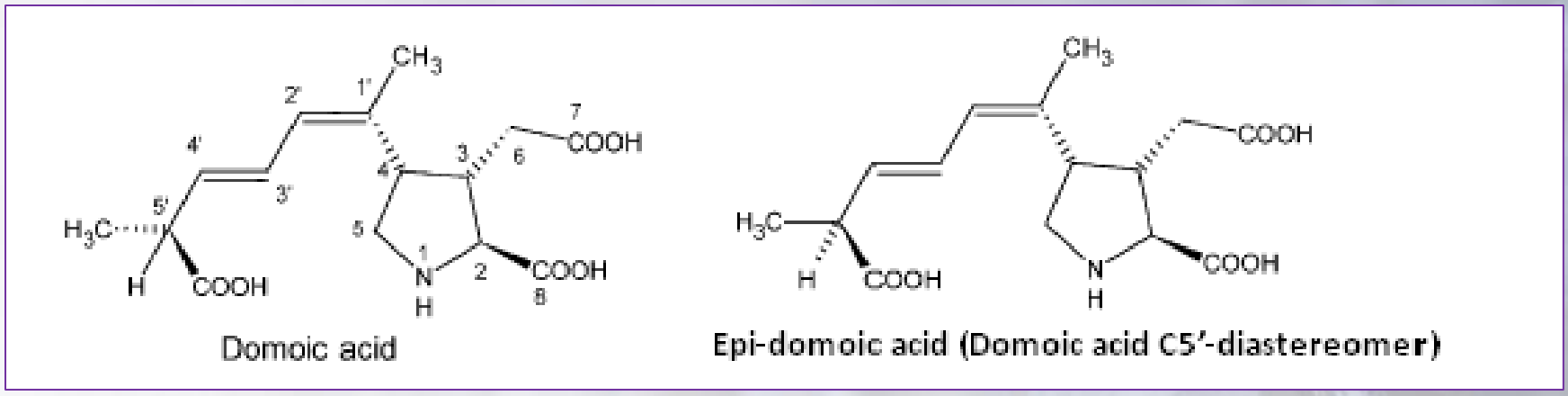
| Advantages | Disadvantages |
|---|---|
| Thoroughly validated | Intensive work, highly trained staff |
| Highly specific (targeted) | Overnight run |
| Accurate concentration assessment | Costs |
| Reproducible | Specific targets – other toxins could be missed |
| Ethically sound | Currently no toxicity screen |

# ASP

*Domoic/epi-domoic acid*

# ASP

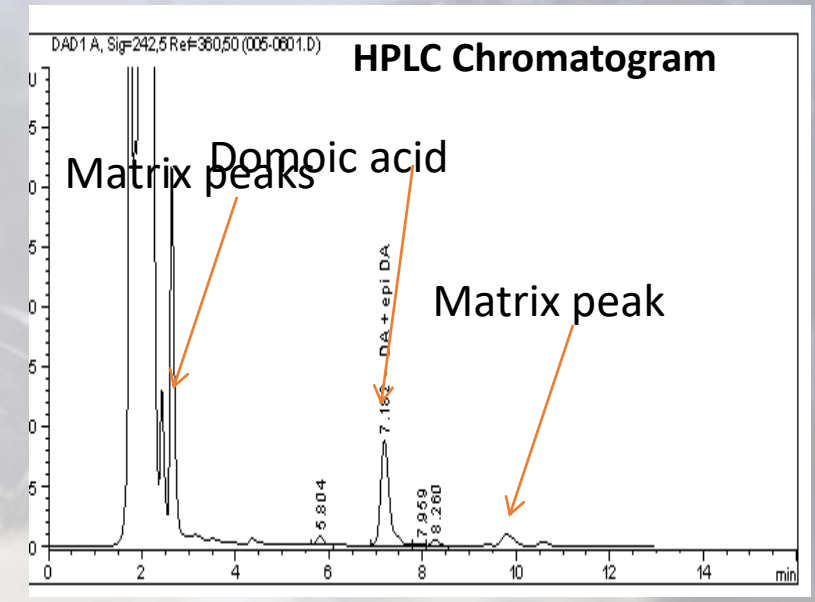
- Domoic acid & epi-domoic acid – total content of whole shellfish or edible part alone




- EU reference method: HPLC-UV
- Shellfish + 50% Methanol extraction
- With or without SPE clean-up
- Very simple, reproducible – no major issues

# HPLC-UV

- EU reference method: HPLC-UV
- Shellfish + 50% Methanol extraction
- Without SPE clean-up
- Very simple, reproducible – no major issues

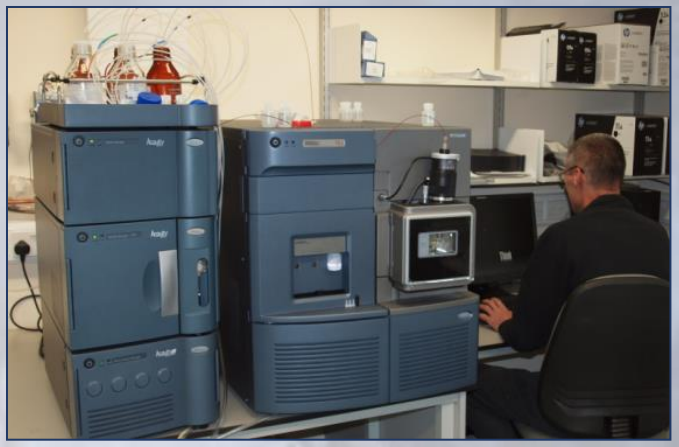
# LTs

*OA, DTXs, YTXs, AZAs, PTXs*

# LC-MS/MS for Lipophilic Toxins

From 1st July 2011

- EU Reference Method
- EU-RL SOP specifies:
  - Aims and scope
  - Extraction and general conditions
  - Performance characteristics



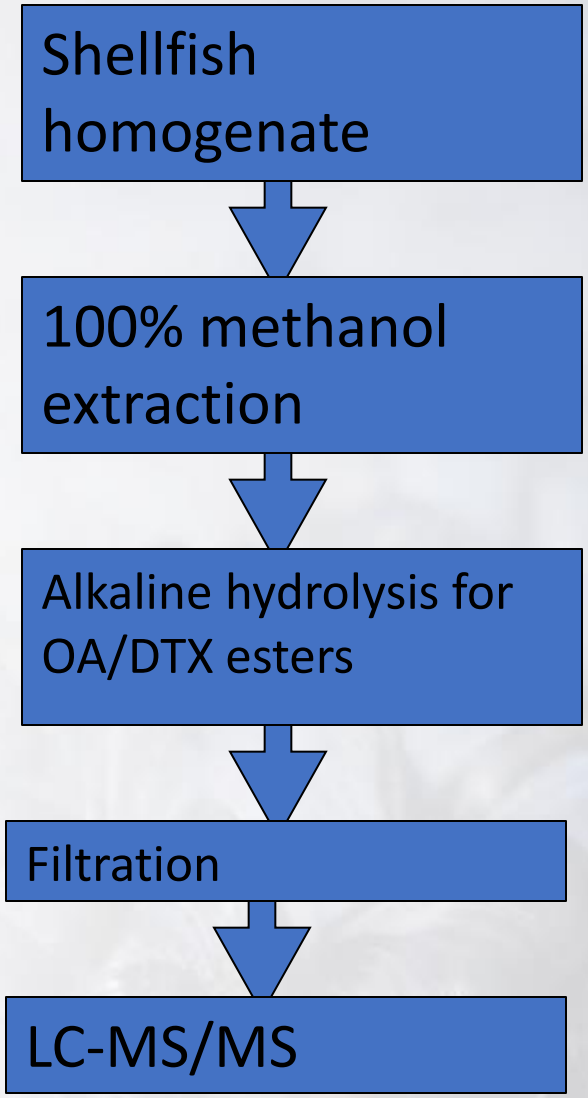
**OA-Group**
- *OA, DTX1, DTX2*
- *Esters of OA-group (DTX3)*
- *PTXs (PTX2, 1, 11)**

**AZA-Group**
- *AZA1, AZA2, AZA3*

**YTX-Group**
- *YTX*
- *Homo-YTX*
- *45 OH YTX*
- *45 OH homo YTX*

**PTXs removed from legislation Sept 2021*

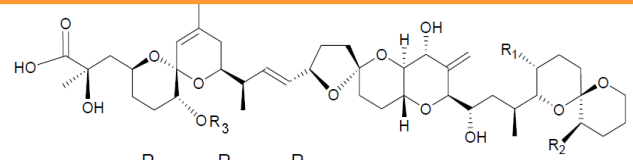
# LT method overview



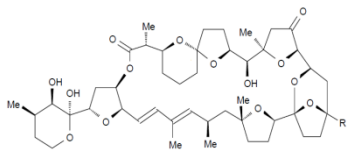

- Results report as:
  - Total OA-group
  - Total AZAs
  - Total YTXs
- Direct determination of toxins available as reference standards
  - Indirect determination of other toxins
- High pH mobile phase (pH 11)
  - Ammonium hydroxide
  - Low pH methods can also be used

# LT LC-MS/MS

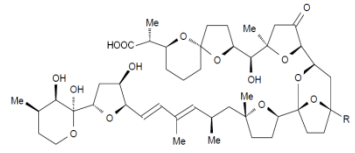
- High proportion of OA/DTXs present as acyl-esters
  - Alkaline hydrolysis to liberate
- +/- switching to encompass all groups
- Now implemented in throughout EU



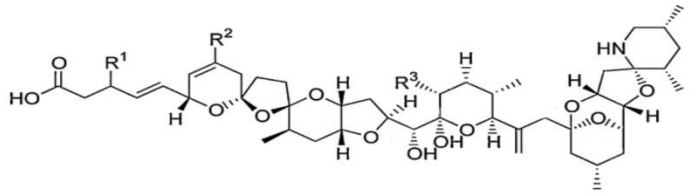
| $R_1$ | $R_2$ | $R_3$ | |
|---|---|---|---|
| $CH_3$ | H | H | Okadaic acid (OA) |
| $CH_3$ | $CH_3$ | H | Dinophysistoxin-1 (DTX1) |
| H | $CH_3$ | H | Dinophysistoxin-2 (DTX2) |
| H | $CH_3$ | acyl | Dinophysistoxin-3 (DTX3) |



| R | |
|---|---|
| $CH_3$ | Pectenotoxin-2 (PTX2) |
| $CH_2OH$ | Pectenotoxin-1 (PTX1) |
| CHO | Pectenotoxin-3 (PTX3) |
| COOH | Pectenotoxin-6 (PTX6) |

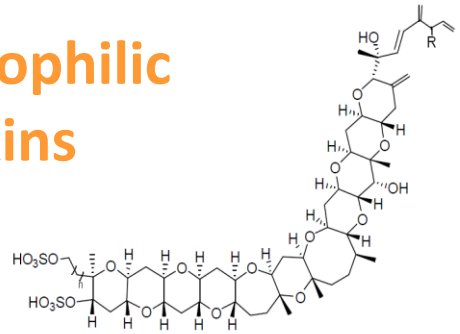


PTX2 seco acid (PTX-2-SA)



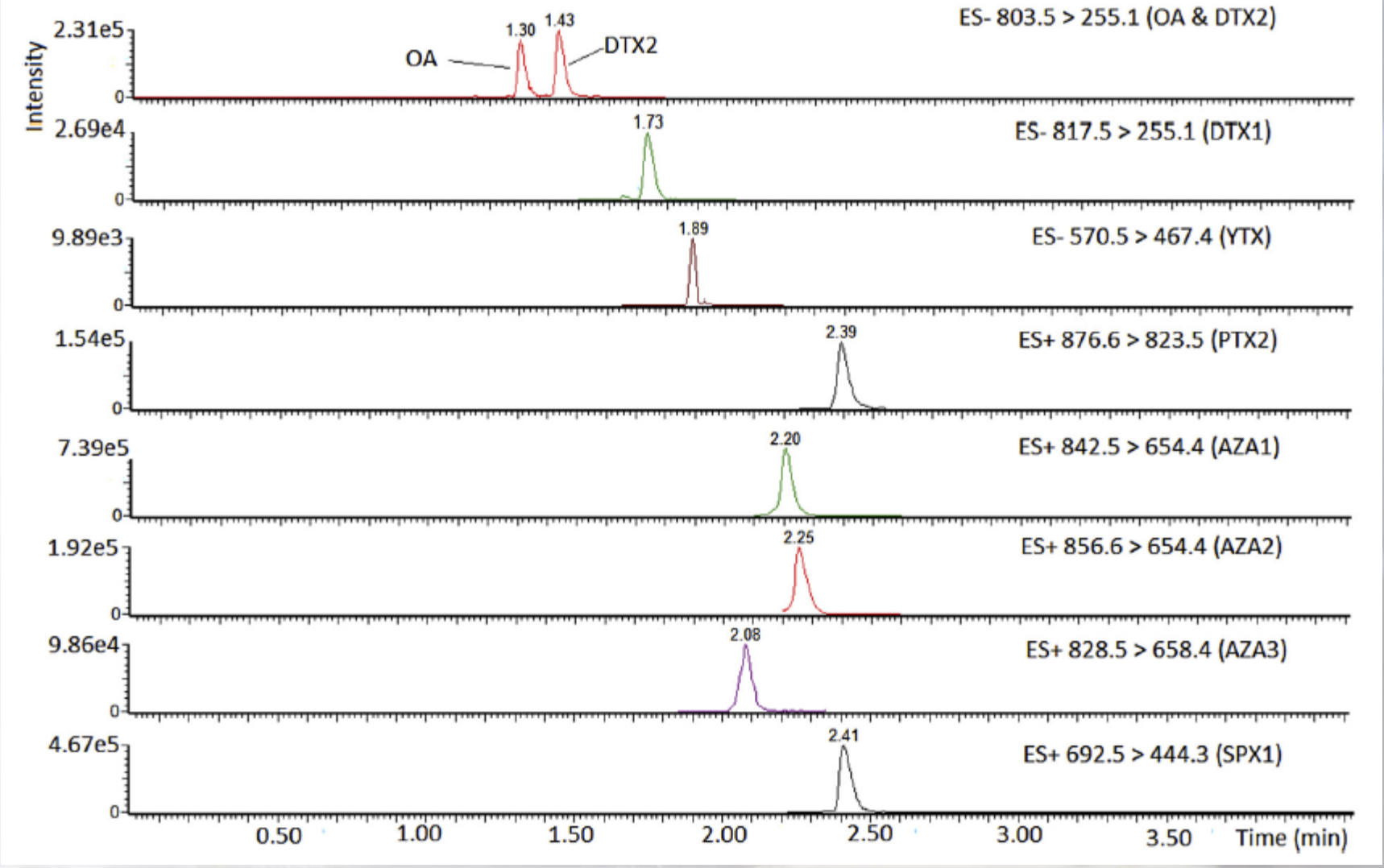
| $R_1$ | $R_2$ | $R_3$ | |
|---|---|---|---|
| H | H | $CH_3$ | Azaspiracid-1 (AZA1) |
| H | $CH_3$ | $CH_3$ | Azaspiracid-2 (AZA2) |

**Lipophilic toxins**



| R | n | |
|---|---|---|
| H | 1 | Yessotoxin (YTX) |
| OH | 1 | 45-Hydroxy-YTX (45-OH-YTX) |
| H | 2 | 1a-homo-yessotoxin (homo-YTX) |
| OH | 2 | 45-Hydroxy-homo-YTX (45-OH-homo-YTX) |

# LT LC-MS/MS



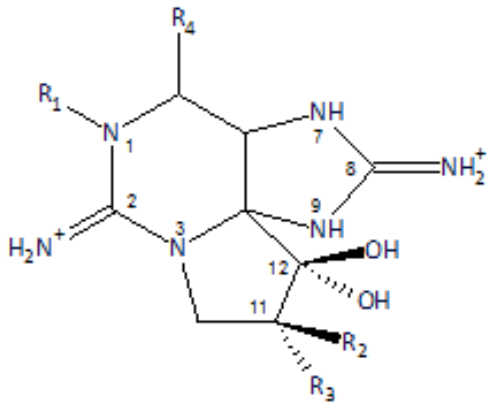

- Now implemented in throughout EU

# PSTs

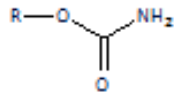
*Saxitoxins*

# PSP toxins

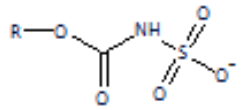
Saxitoxin derivatives




OCONH$_2$:




OCONHSO$_3^-$:

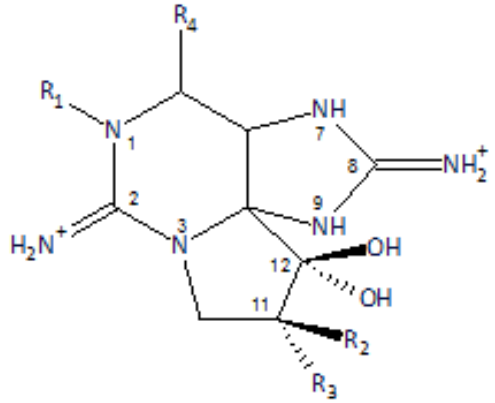



| Group (Charge state) | Analogue | R1 | R2 | R3 | R4 |
|---|---|---|---|---|---|
| C toxins (0) | C1 | H | H | $OSO_3^-$ | $OCONHSO_3^-$ |
| | C2 | H | $OSO_3^-$ | H | $OCONHSO_3^-$ |
| | C3 | OH | H | $OSO_3^-$ | $OCONHSO_3^-$ |
| | C4 | OH | $OSO_3^-$ | H | $OCONHSO_3^-$ |
| GTXs (+1) | dcGTX2 | H | H | $OSO_3^-$ | OH |
| | dcGTX2 | H | $OSO_3^-$ | H | OH |
| | dcGTX1 | OH | H | $OSO_3^-$ | OH |
| | dcGTX4 | OH | $OSO_3^-$ | H | OH |
| | GTX2 | H | H | $OSO_3^-$ | $OCONH_2$ |
| | GTX3 | H | $OSO_3^-$ | H | $OCONH_2$ |
| | GTX1 | OH | H | $OSO_3^-$ | $OCONH_2$ |
| | GTX4 | OH | $OSO_3^-$ | H | $OCONH_2$ |
| | GTX5 (B1) | H | H | H | $OCONHSO_3^-$ |
| | GTX6 (B2) | OH | H | H | $OCONHSO_3^-$ |
| | M1α | H | H | OH | $OCONHSO_3^-$ |
| | M1β | H | OH | H | $OCONHSO_3^-$ |
| | M3 | H | OH | OH | $OCONHSO_3^-$ |
| STXs (+2) | dcSTX | H | H | H | H |
| | dcSTX | H | H | H | OH |
| | dcNEO | OH | H | H | OH |
| | STX | H | H | H | $OCONH_2$ |
| | NEO | OH | H | H | $OCONH_2$ |
| | M2α | H | H | OH | $OCONH_2$ |
| | M2β | H | OH | H | $OCONH_2$ |
| | M4 | H | OH | OH | $OCONH_2$ |

- N-hydroxyl
  - Carbamate NEO, GTX1&4
  - Decarbamoyl dcNEO, dcGTX1&4
  - N-sulfocarbamate GTX6, C3&4
- Non N-hydroxyl
  - STX, GTX2&3, dcSTX, dcGTX2&3, GTX5, C1&2
- Others
  - M toxins, GC toxins and more…
- All have different toxicities; TEF of some still unknown

# PSP toxins

Saxitoxin derivatives




| Group (Charge state) | Analogue | R1 | R2 | R3 | R4 |
|---|---|---|---|---|---|
| C toxins (0) | C1 | H | H | $OSO_3^-$ | $OCONHSO_3^-$ |
| | C2 | H | $OSO_3^-$ | H | $OCONHSO_3^-$ |
| | C3 | OH | H | $OSO_3^-$ | $OCONHSO_3^-$ |
| | C4 | OH | $OSO_3^-$ | H | $OCONHSO_3^-$ |
| GTXs (+1) | dcGTX2 | H | H | $OSO_3^-$ | OH |
| | dcGTX2 | H | $OSO_3^-$ | H | OH |
| | dcGTX1 | OH | H | $OSO_3^-$ | OH |
| | dcGTX4 | OH | $OSO_3^-$ | H | OH |
| | GTX2 | H | H | $OSO_3^-$ | $OCONH_2$ |
| | GTX3 | H | $OSO_3^-$ | H | $OCONH_2$ |
| | GTX1 | OH | H | $OSO_3^-$ | $OCONH_2$ |
| | GTX4 | OH | $OSO_3^-$ | H | $OCONH_2$ |
| | GTX5 (B1) | H | H | H | $OCONHSO_3^-$ |
| | GTX6 (B2) | OH | H | H | $OCONHSO_3^-$ |
| | M1α | H | H | OH | $OCONHSO_3^-$ |
| | | | | | $OCONHSO_3^-$ |
| | | | | | $OCONHSO_3^-$ |
| | | | | | H |
| | | | | | OH |
| | | | | | OH |
| | | | | | $OCONH_2$ |
| | | | | | $OCONH_2$ |
| | | | | | $OCONH_2$ |
| | | | | | $OCONH_2$ |
| | | | | | $OCONH_2$ |

Thankfully: PSTs commonly occurring in naturally contaminated shellfish are available as standards **and** most have fairly well described TEFs

- N-hydroxyl
  - Carbamate NEO,
  - Decarbamoyl dcNEO, dcGTX1&4
  - N-sulfocarbamate GTX6, C3&4
- Non N-hydroxyl
  - STX, GTX2&3, dcSTX, dcGTX2&3, GTX5, C1&2
- Others
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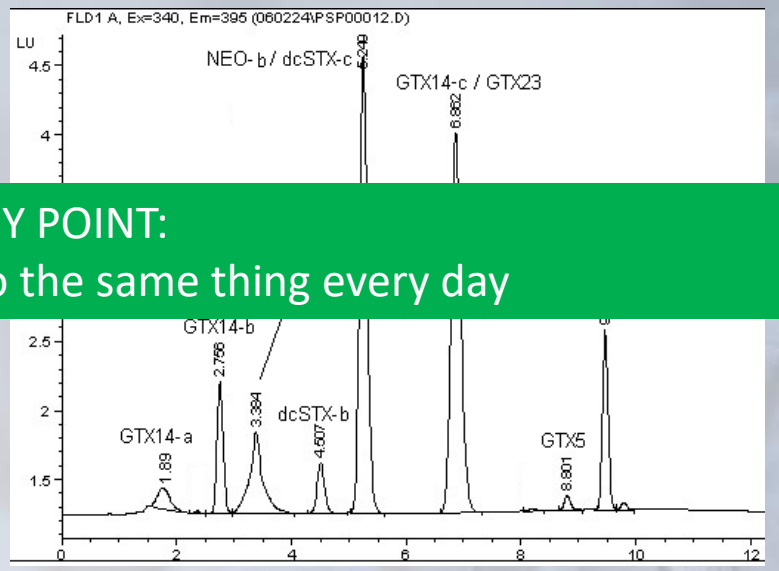
# PSP LC-FLD

(AOAC 2005.06 OMA)




**KEY POINT:**
**Do the same thing every day**

- Shellfish homogenate
  - Extraction (1% Acetic acid)
    - C18 SPE clean-up / pH adj
      - Periodate ox (screen)
        - HPLC-FLD
          - Peroxide oxidation
            - HPLC-FLD
              - STX, dcSTX, GTX2/3, GTX5, C1/2, dcGTX2,3
          - Ion exchange SPE (fractionation)
            - Fraction#1 (C toxins)
              - Perox.
                - C1/2
              - Period
                - C3/4
            - Fraction#2 (GTX1/4, GTX2/3, GTX5, GTX6)
              - Perox.
                - GTX2/3, GTX5
              - Period
                - HPLC-FLD
                - GTX1/4 GTX6
            - Fraction#3 (STX, NEO, dcNEO, dcSTX)
              - Perox.
                - STX, dcSTX
              - Period
                - HPLC-FLD
                - NEO, dcNEO
          - Unoxidised Non-toxic co-extractives
            - HPLC-FLD

# Current approach

- Periodate screen of every sample
- Semi-quantitative “toxicity” reported
- Only samples >400 µg STX eq/kg are subjected to full clean up and quantitation
- All others reported as either:
  - Not detected
  - Detected (< 400)
- Reduces requirement for quantitation significantly

# Validation and Implementation

# Validation of Methods

## Not an easy, quick or cheap process:

- Initial testing of method
- Assessment of issues
- Resolve practical issues and pitfalls
- In-house validation to define performance
- Comparison with other methods
- Define implementation approaches
- Implement

**Validation**
Selectivity
LOD/LOQ (screen & quant)
Linearity and range
Accuracy (CRM)
Toxin recovery
Precision (short, medium, long term)
Ruggedness
Uncertainty of measurement

## To be done for each species

IUPAC
International Union of Pure and Applied Chemistry

# Implementation of “new” methods

In EU: Process is time-consuming:

- Method developed and single-lab validated:
  - Must follow full EC / IUPAC guidelines
  - Demonstrate “equivalence” with current ref method
- Formal multi-lab collaborative study
  - Following specific guidelines (*e.g*. AOAC)
- Publication as Official Method (*e.g*. AOAC, CEN)
- Method acceptable within EU legislation
- Approval by Competent Authority and COT
- Accreditation to ISO17025

**Implementation now may be possible**

# Practical Application of Methods

**Key Points**

- ISO 17025
- Highly trained analysts
- Robust instrumentation
- Automated processes
- Risk awareness, mitigation and contingency
- Availability of reference materials

- Internal Quality Control
- Positive controls
- Blanks
- Calibrations
- Calibration checks
- Trend analysis

- External Quality Assurance
- Proficiency testing schemes
- Ring trials
- External materials

Cefas

# Aquatic toxin mitigation – known/regulated

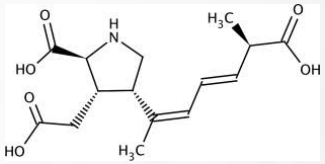
2000 - 2010

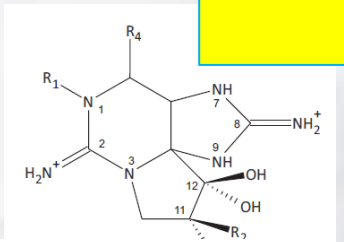
PSP – using MBA
DSP – using MBA
ASP – HPLC-UV

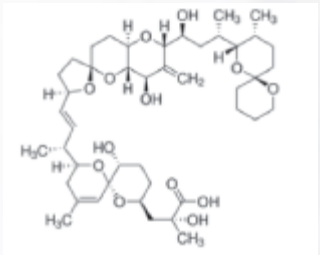
2010 -

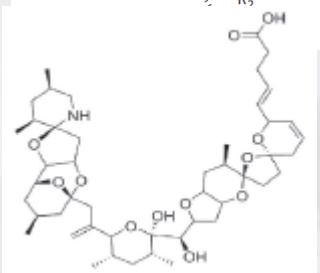
PSP – HPLC-FLD
LTs – LC-MS/MS
ASP – HPLC-UV

**Targeted detection of known, regulated toxins only**

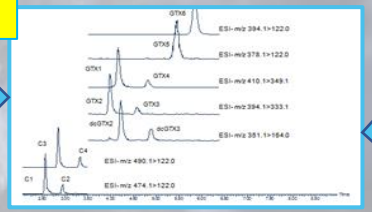








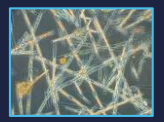
Official control

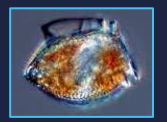


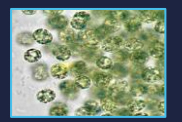
End product


Centre for Environment Fisheries & Aquaculture Science

Cefas







# End product testing

LFIA



ELISA/PP2A

30.4.2004 EN Official Journal of the European Union L 155/206

**REGULATION (EC) No 854/2004 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 29 April 2004**

laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption

Food businesses required under EU law to ensure that shellfish placed on the market are safe for consumption and do not exceed the MPLs stipulated in the EC regulations

| Supplier | Kit type | Brief summary of findings |
|---|---|---|
| ZeuLab | PP2A | • In general – good qualitative indication of toxicity from most kits<br>• Variable accuracies of quantitation<br>  • Linear range inappropriate for some ELISA<br>• Low false +ve – for most kits<br>• Low false –ve – for most kits<br>• Scan value from LFIA very useful<br>• In combination with portability – LFIA powerful and flexible tools<br><br>• Some issues still need investigation<br>• More assessments using test kit of choice |
| BiooScientific | ELISA | |
| Abraxis | ELISA | |
| Beacon | ELISA | |
| Europroxima | ELISA | |
| R-Biopharm | ELISA | |
| Scotia | LFIA | |
| Neogen | LFIA | |

# Future

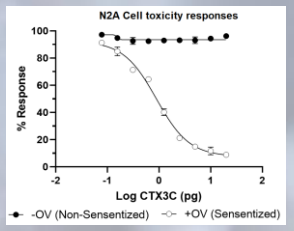
2000 - 2010

2010 -

Targeted methods risk missing new hazards

Emerging diagnostics

2020 -




Protection from emerging hazards:

- Ethical bioassays
- Untargeted toxin screening
- Metabolomic biomarkers
- Genetic methods
- Toxicology catch-up

Combine to develop new targeted, quantitative approaches

Neuroblastoma cell toxicity assay for Na channel toxins

qPCR for 18s rDNA detection of PST-producing phytoplankton species

High resolution LC-MS for accurate mass detection

Nanopore sequencing for HAB sp.

# Thank you for listening

**Dr Andrew D. Turner CChem CSci**

Principal Chemist Food Safety Group

Topic Leader Natural Aquatic Toxins

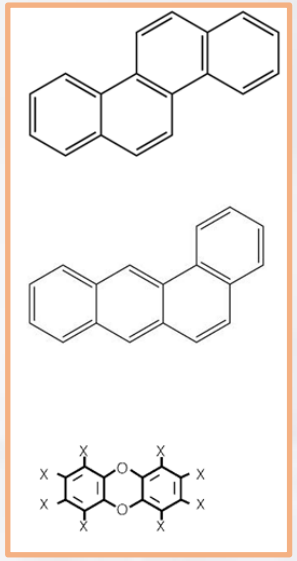
FAO Reference Centre Marine Toxins Advisor

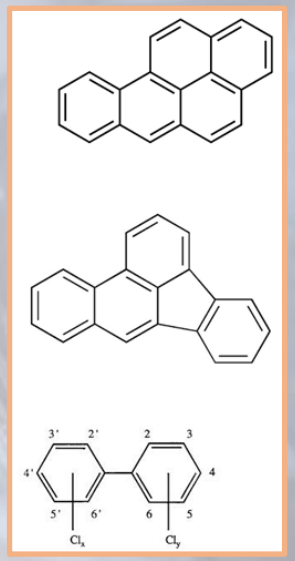
Andrew.turner@cefas.co.uk

Cefas INTERNATIONAL CENTRES OF EXCELLENCE

Food and Agriculture Organization of the United Nations

# Sampling and analysis of shellfish for chemical contaminants



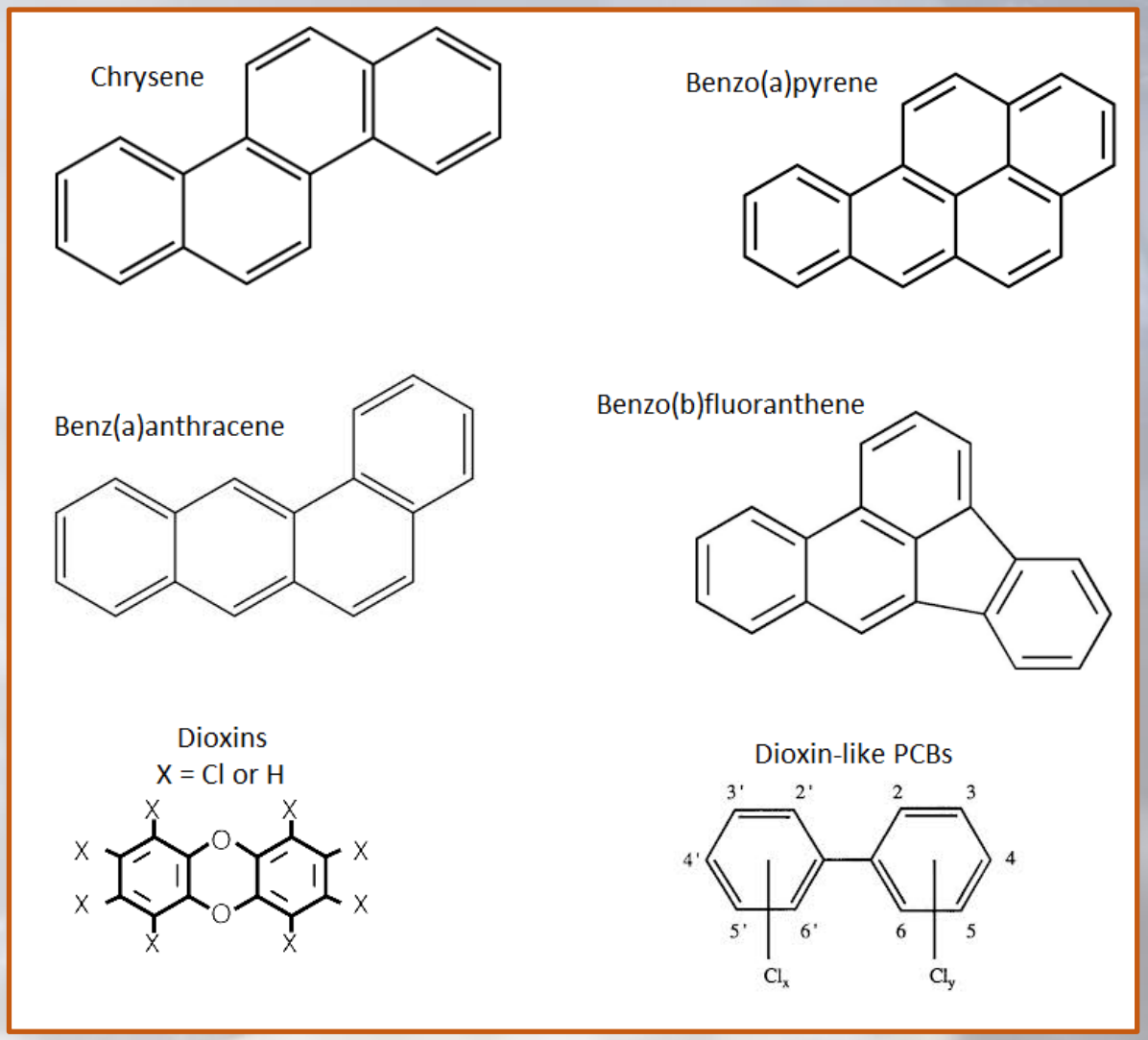


# Chemical contaminant regulations

- **EU Regulations specify:**
  - Maximum permitted levels
  - Sampling criteria
  - Analytical methods & performance characteristics

# Chemicals tested

- Metals
  - Pb, Cd, Hg
- PAHs
  - 4 compounds
- Dioxins and dioxin-like PCBs – all organochlorines



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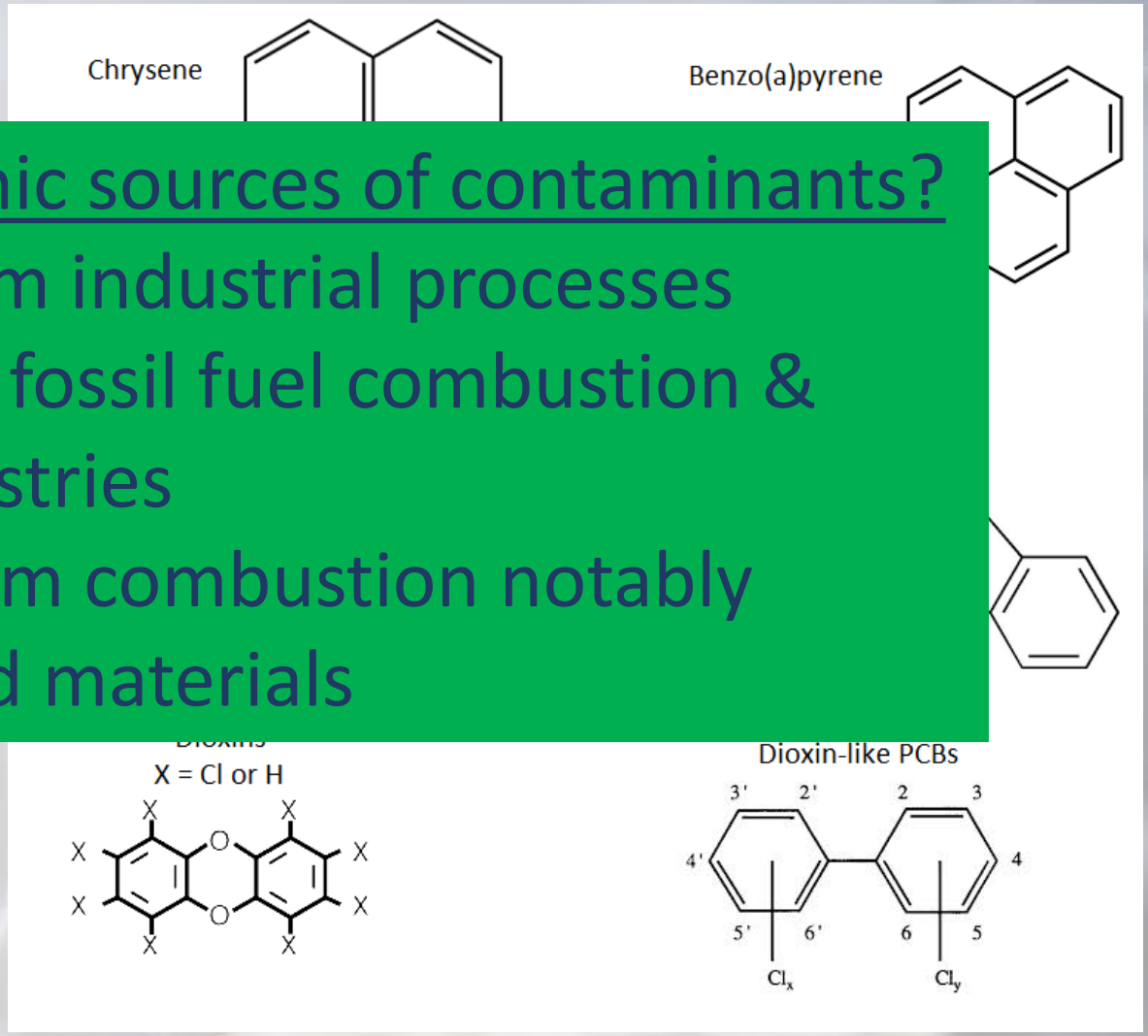



## Anthropogenic sources of contaminants?

- Metals from industrial processes
- PAHs from fossil fuel combustion & other industries
- Dioxins from combustion notably chlorinated materials

# Toxicity

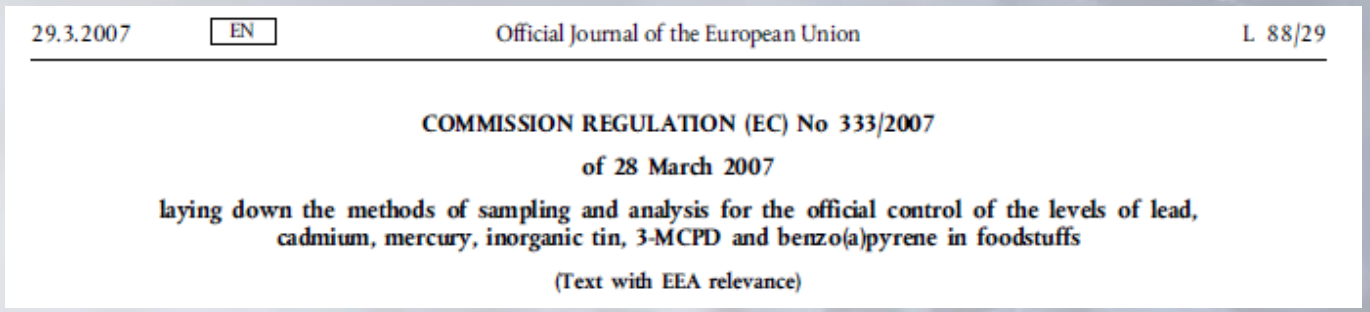
- **Metals** poisoning – well known high acute and chronic toxicity
- **PAHs** – Acute (D&V, skin irritation, confusion), chronic (eye/organ damage, breathing problems) + carcinogen, genotoxic, immunotoxic
- **Dioxins** – Acute (skin irritation, pain), known carcinogens & links to learning disabilities, reproductive effects & immunotoxic

# Regulatory limits

- ***Metals***
- Lead (Pb) – 1.5 mg/kg
- Cadmium (Cd) – 1.0 mg/kg
- Mercury (Hg) – 0.50 mg/kg

- ***PAHs***
- 5.0 µg/kg for Benzo(a)pyrene
- 30.0 µg/kg for sum of 4 PAHs:
  - Benzo(a)pyrene
  - Benz(a)anthracene
  - Benzo(b)fluoranthene
  - Chrysene

- ***Dioxins***
- Sum of dioxins (WHOPCDD/F-TEQ) = 3.5 pg/g
- Sum of dioxins and dioxin-like PCBs (WHOPCDD/F-PCB-TEQ) = 6.5 pg/g



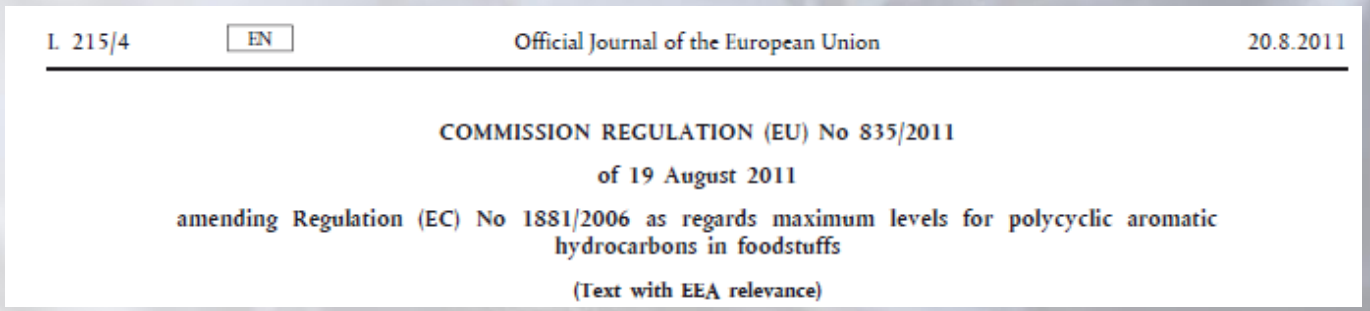
29.3.2007 EN Official Journal of the European Union L 88/29

COMMISSION REGULATION (EC) No 333/2007

of 28 March 2007

laying down the methods of sampling and analysis for the official control of the levels of lead, cadmium, mercury, inorganic tin, 3-MCPD and benzo(a)pyrene in foodstuffs

(Text with EEA relevance)



L 215/4 EN Official Journal of the European Union 20.8.2011

COMMISSION REGULATION (EU) No 835/2011

of 19 August 2011

amending Regulation (EC) No 1881/2006 as regards maximum levels for polycyclic aromatic hydrocarbons in foodstuffs

(Text with EEA relevance)

# Representative samples

- Sampling frequency – determined by FSA/FSS RA – currently once per year
  - Except where samples non-compliant (or close)
- Samples to come from selected classified area as per FSA/FSS risk assessment
- Sampling locations defined by FSA/FSS, usually to match toxin or E.coli monitoring point
- Assessed pre-spawning (higher contaminant levels)

# Sample weights

- 100g homogenised tissue minimum (PAHs/metals only)
- 500g tissue for full suite (+ Dioxins)
- Guidance provided to LAs
- Cool box packing advice also provided

| Species | Approx number or weight in shell to provide **100g** flesh for PAH/metal analysis (*nb 100g is required as minimum)* | Approx number or weight in shell to provide **500g** flesh for full suite analysis |
|---|---|---|
| Oysters (Crassostrea gigas and Ostrea edulis) | 16-20 | 80-100 |
| Hard Clams | 16-20 | 80-100 |
| Surf clams | 16-25 or 1 kg | 80-125 |
| Rope grown mussels | 60 or 700g | 300 or 3kg |
| Shore mussels | 800g | 4kg |
| Cockles | 100 or 700g | 500 or 3kg |

- Cefas provide transport boxes + pre-paid delivery labels & forms

# Instrument methods

- ICPMS for metals
- HRGC-LRMS for PAHs
- HRGC-HRMS for dioxins/PCBs

Waters Micromass AutoSpec Premier.

# Quality

- All testing using standard methods
- All methods formally validated
- Accredited to ISO17025
- ISO17025 auditors inspect annually
- Active involvement in proficiency testing for quality assurance
- PT results provided to customer annually

# Outcome from results

- FSA/FSS & LAs informed when results above compliance limits (or close)
- Further sampling may be required if results exceeding limits

# Overall

- Chemical detection methods provide powerful tools for the protection of shellfish consumers from contaminated shellfish products

- Methods need to be tested and validated in each lab for the species of relevance

- Labs must participate in IQC and EQA procedures routinely

- Need to be aware of the potential for “new” or “emerging” toxin threats, now and in the future – more data needs to be generated

- Ideally, new biological assays to complement chemical detection tools