

# Application of bioassays in routine testing

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# 1957: chickens discover dioxins



- Millions of dead and diseased chickens in US
- Chicken oedema disease
- After ten years dioxins identified as toxic agent
- Source: fat scrapings from cow hides that were treated with polychlorophenols
- Similar incident in 1969 in North Carolina due to wastewater from pesticide plant

# The Belgian dioxin crisis in 1999



# Dioxin and PCB levels in feed, chicken and egg

Sample	Dioxins <sup>1</sup> (pg WHO-TEQ/g)	Planar PCBs <sup>2</sup> (pg WHO-TEQ/g)	PCBs <sup>3</sup> (µg/g)
Animal feed	782	361	32
Chicken fat	958	453	37
Egg fat	685	ND	35

Source: >160 kg  
PCB-oil !

<sup>1</sup> Background levels below 5 pg WHO-TEQ/g fat.

<sup>2</sup> Planar PCBs reflects the sum of PCBs 126, 169 and 77.

<sup>3</sup> PCB levels reflect the sum of PCBs 28, 52, 101, 118, 138, 153, 180, which account for about 30% of the dioxins in the case of a PCB-mixture of Arochlors 1254 and 1260.

# Incidents in the food chain



- Dioxins in feed and food
  - USA 1957, Japan (1967), Taiwan (1979), Belgium (1999)
- Aflatoxins in turkey feed (turkey-X-disease, 1960)
- PBBs in bovine feed (Michigan, 1973)
- Medroxyprogesteron acetate/estradiol in feed (2002)
- Poisoning Victor Yuchenko (2004)
- Melamine in petfood and later milk powder (2008)
- Supplements including tea (various cases)

# Discovery

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- Discovery based on health effects
- Leading to measures
  - Including setting of limits
- Including increased monitoring
  - Use of chemical methods
  - Only occasionally use of bioassays
- Identification required for legal follow-up
- But chemical analysis is dedicated
  - We only look for “knowns”, not “for unknowns”
- If added on purpose, use of “unknowns” is preferred



# Types of bioassays



- Humans (to be prevented of course)
- Animals (birds in mines, mice/rats for marine toxins)
- Bacteria (growth inhibition by antibiotics)
- Yeast cells (reporter gene assays for hormones)
- Mammalian cells
  - Based on known effects (cell death, growth up/down, up-regulation enzyme)
  - Transfected cells (reporter gene assays)
  - Broad effects based on gene expression

# Application of bioassays

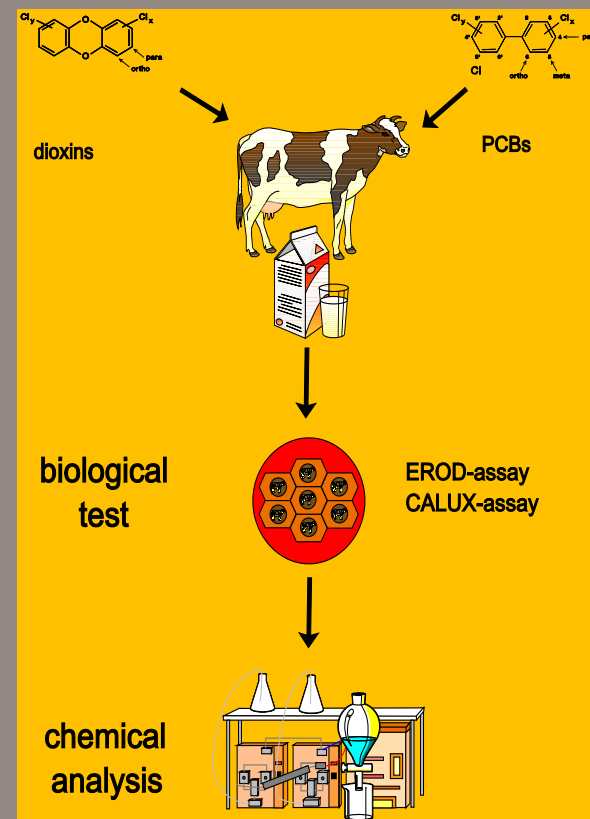
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- Regarded as screening assay
- Used for selection of samples
  - However, most samples normally negative
  - Negative or suspected
  - Estimation of level possible but not required
- Also may detect other compounds with same effects
  - False-positive or indication for novel risk?

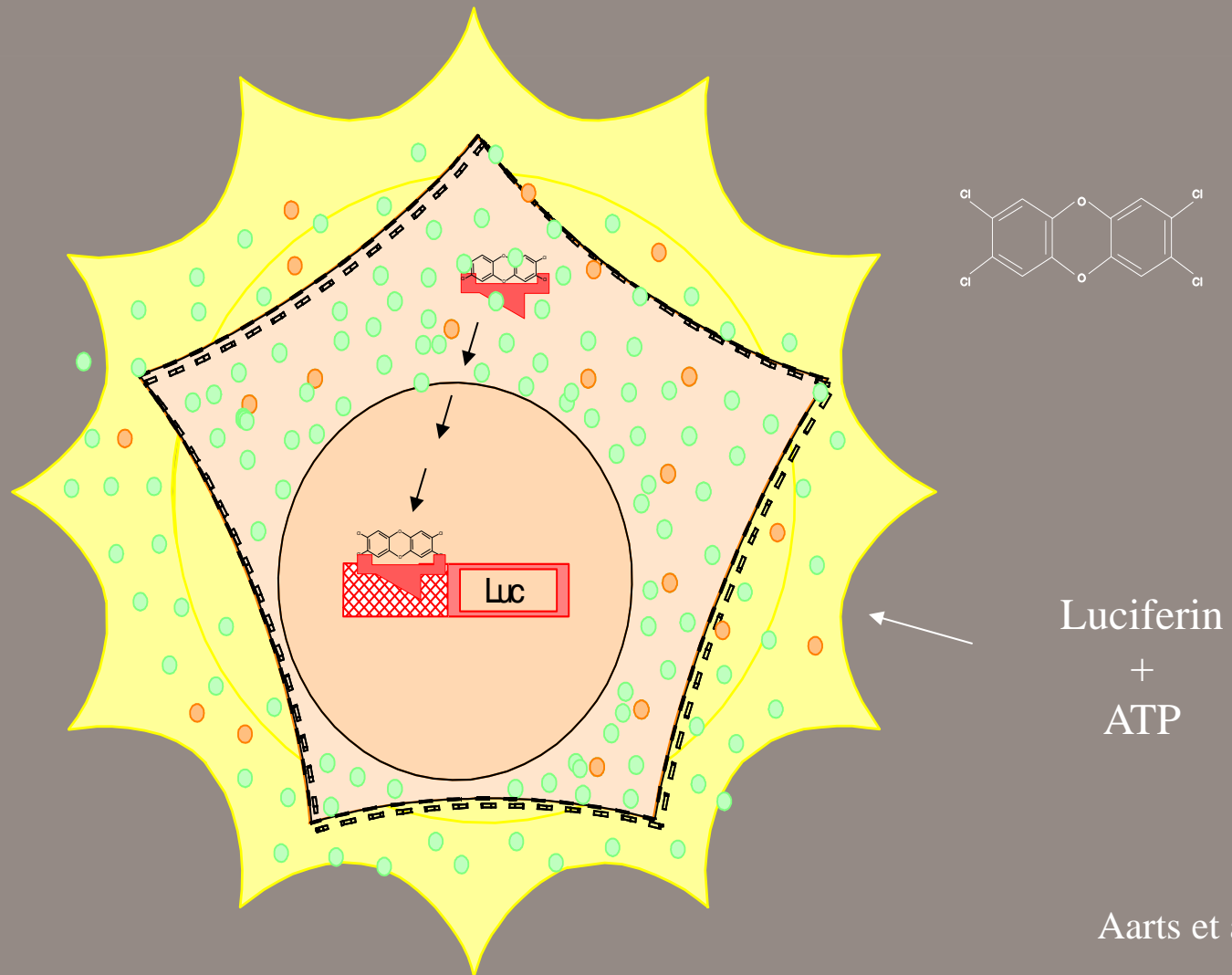


# Dioxins: GC/HRMS and/or bioassay

- DR CALUX: screening
  - removal negative samples
  - confirmation suspects
- GC/HRMS: confirmation

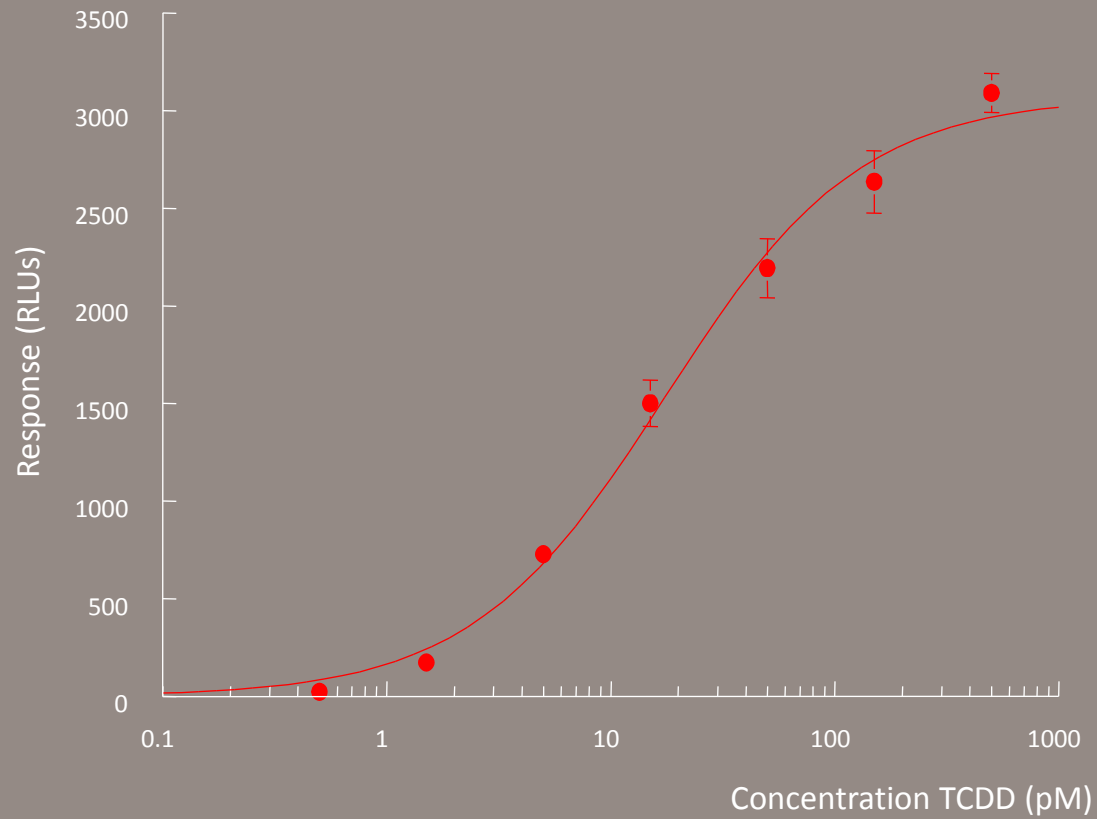


# CALUX screening assay



Aarts et al. 1993

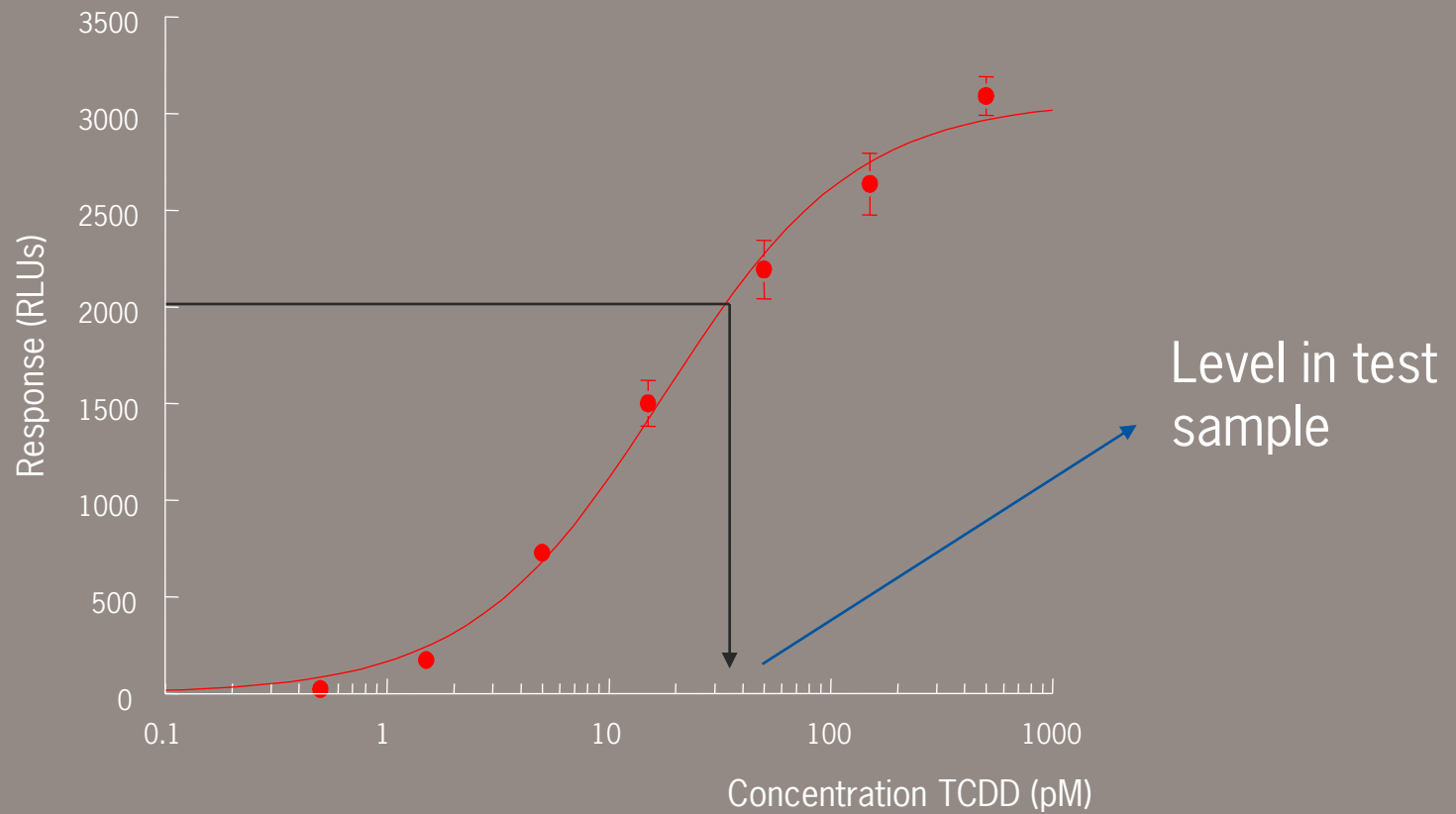
# Dose-related response



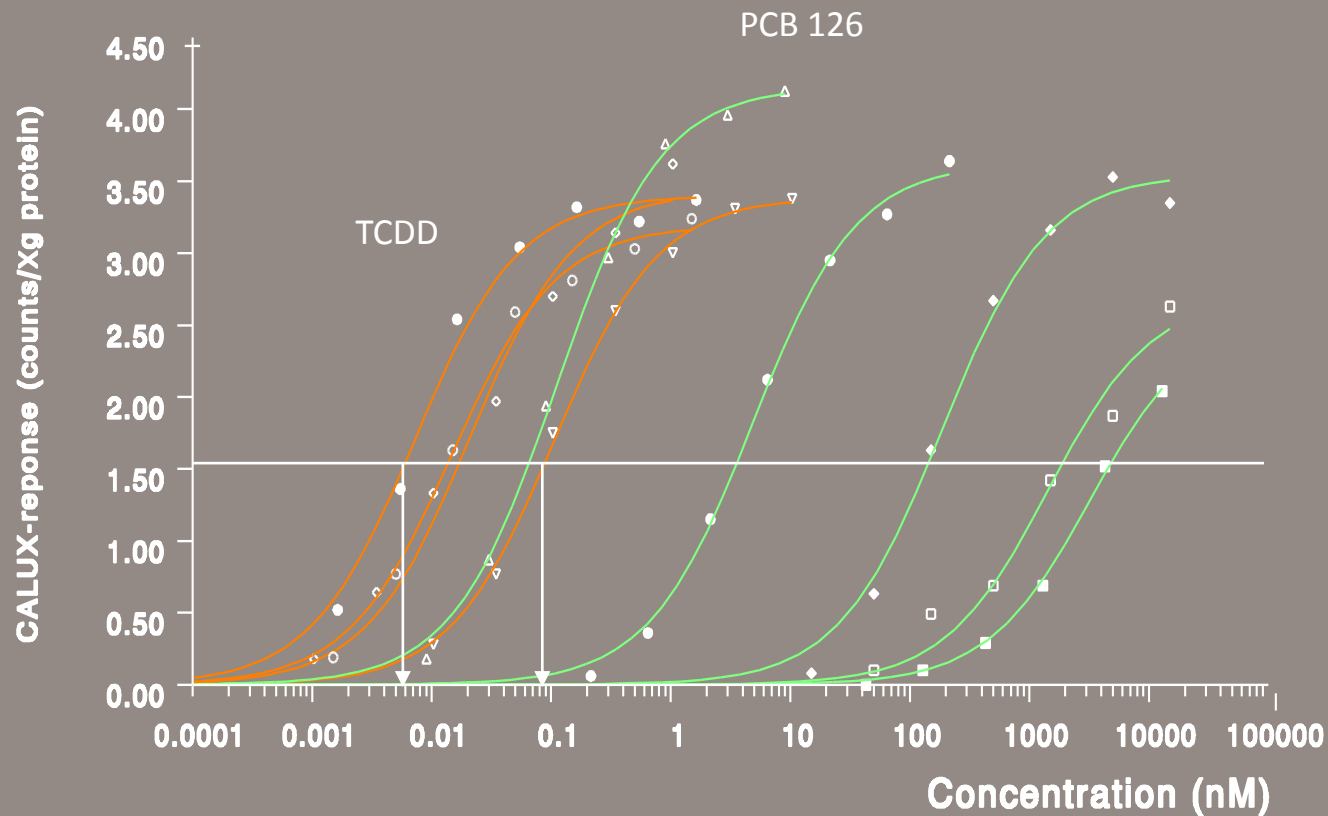
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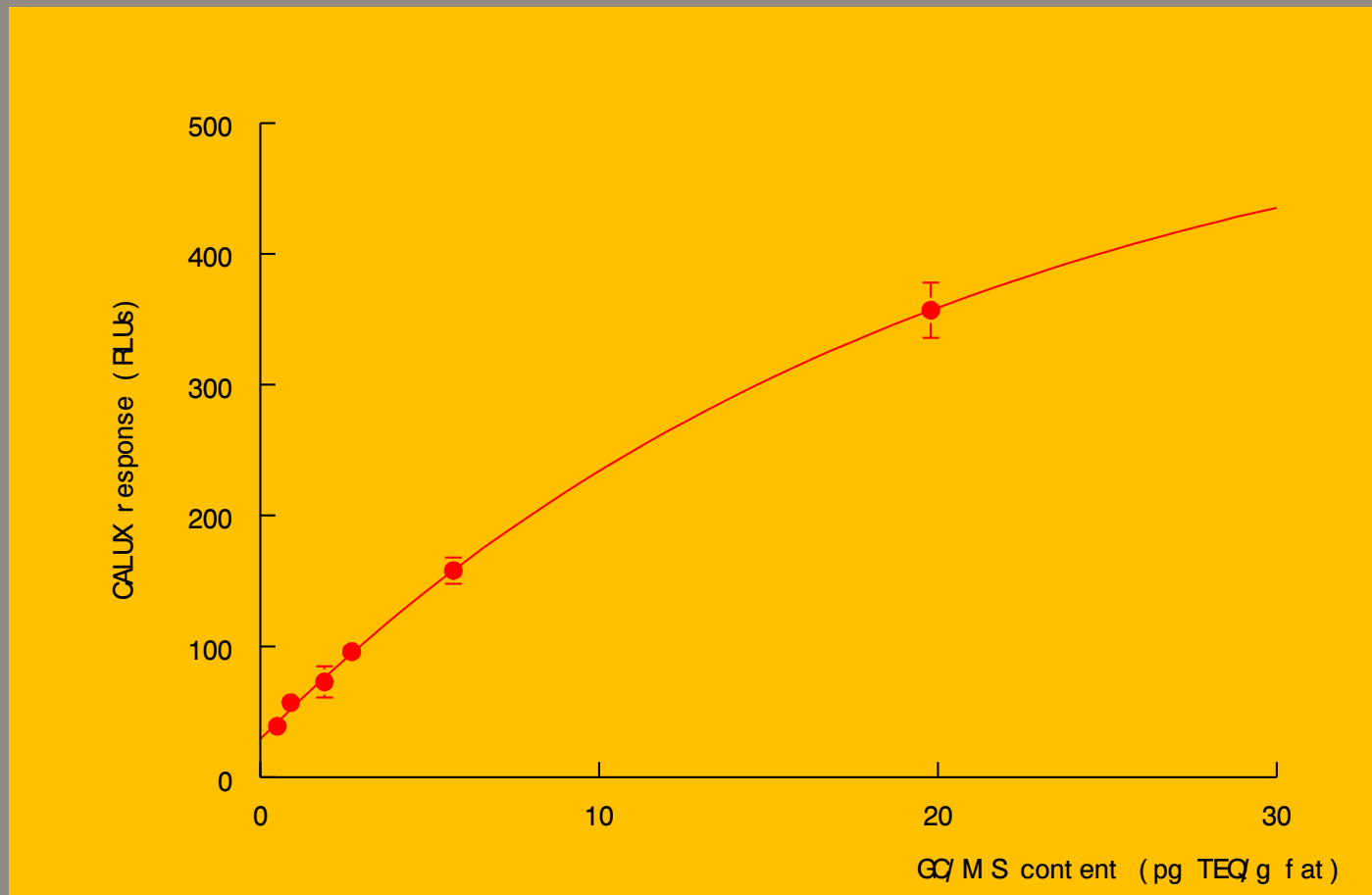
# Estimation of level in sample



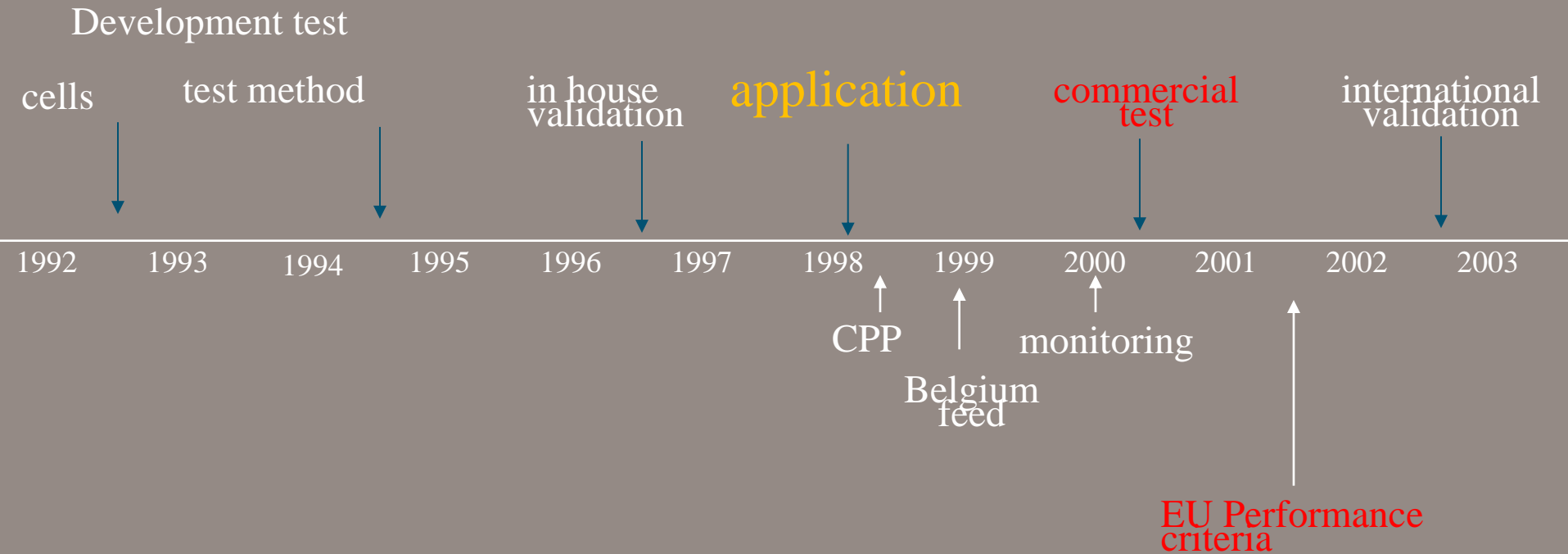
# Dose-response curves dioxins and dl-PCBs



# Calibration curve of reference samples (butter fat)



# History of the CALUX-assay





# Performance criteria

- Criteria proposed by experts
  - Papers presented at Dioxin 2001 South Korea
- Use to prepare Commission Directives 2002/69/EC (food) and 2002/70/EC (feed)
- Upgraded to Commission Regulations EC (No) 1883/2006 (food) and 152/2009 (feed)
- Recently replaced by Commission Regulations EC (No) 252/2012 (food) and 278/2012 (feed)
  - based on the work of expert group EURL/NRL

# Screening/quantitative approach

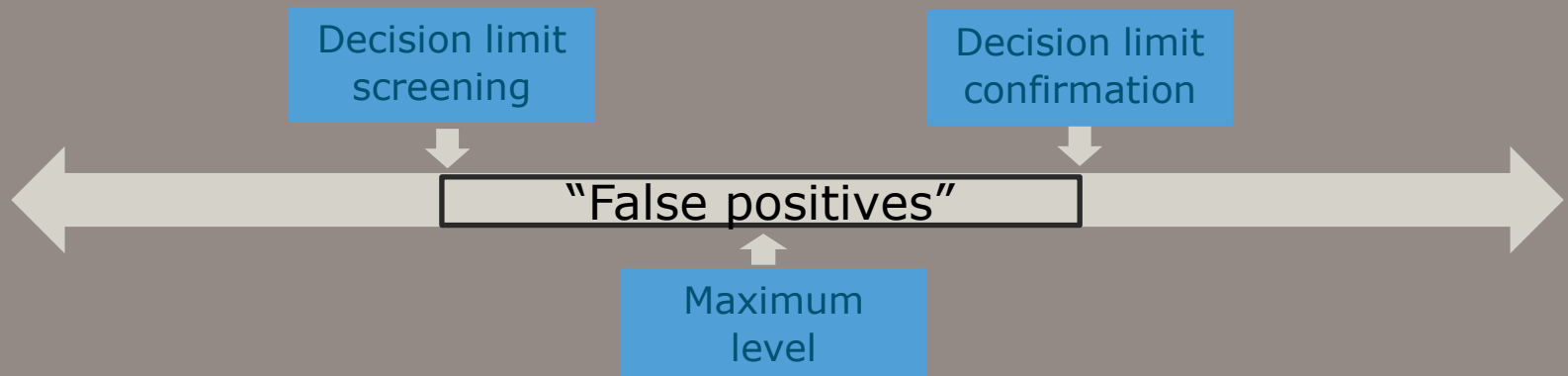
- CALUX is a screening method (yes/no answer)
- Estimation may be given; support confirmatory analysis
- Should be in BEQs and not TEQs
  - REPs not identical to TEFs
  - Also compounds w/o TEF may show response
  - Screening result should be recognizable
- Estimation of level may be based on:
  - standard curve (eg TCDD/PCB 126)
  - Set of reference samples

# False-compliant rate

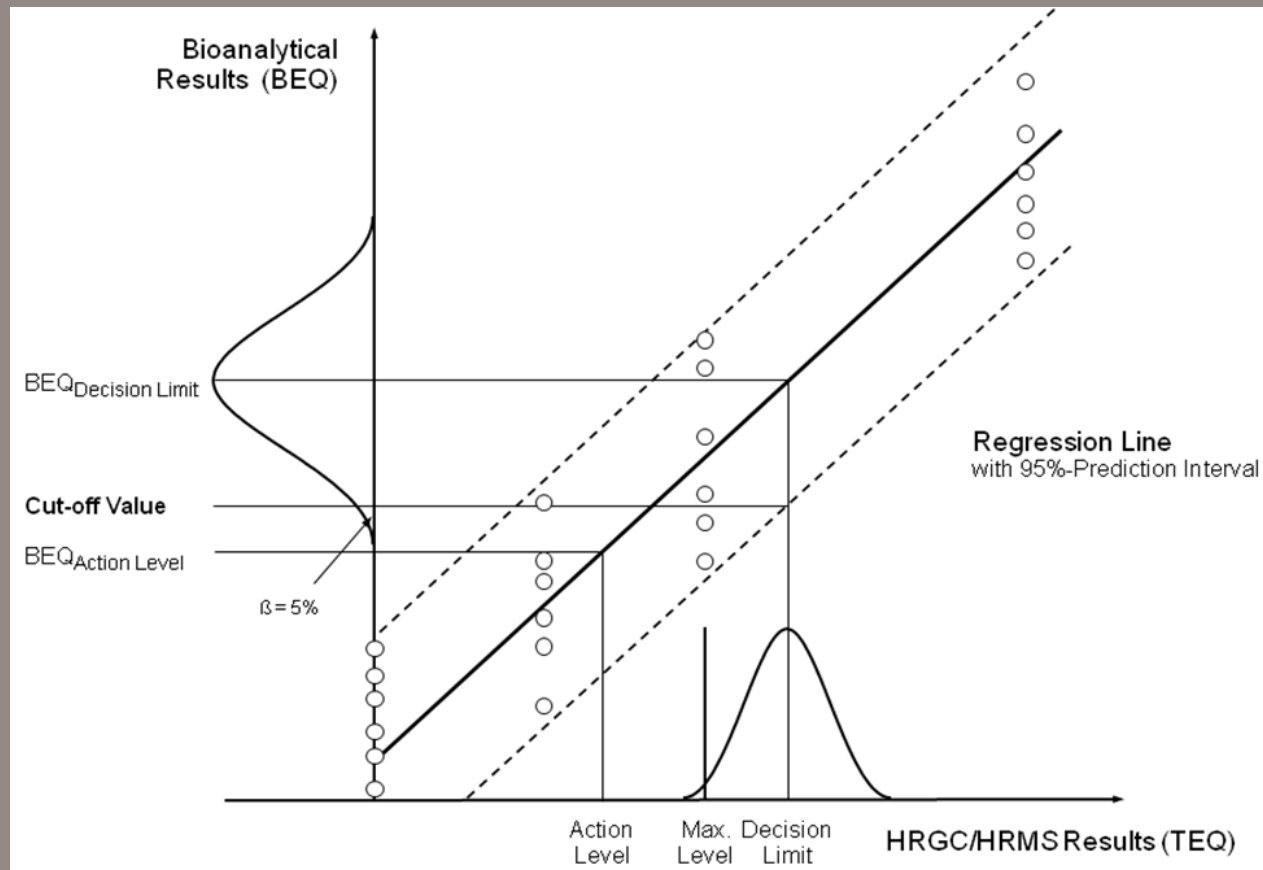
- Previously set at 1%
  - Difficult to prove compliance
  - Hundreds of positive samples need to be analyzed
  - Not clear if towards ML or AL
- In other areas 5% is used (EC 2002/657)
- Therefore changed to 5%
- Refers to maximum limit, not action limit
  - Performance towards action limit should be evaluated

# Setting of cut-off levels

- Screening should not miss positive samples
  - Chance less than 5%
- Confirmation should not falsely decide on positive result
  - Chance less than 5%



# Setting of cut-off levels for screening



Proposal: use 2/3 ML ( $\approx$ AL) as cut-off for screening

# Practical performance



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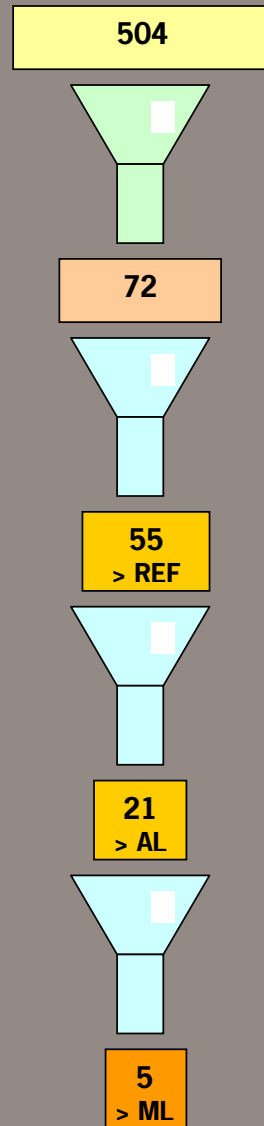
# Performance at RIKILT

	ALs <sup>1</sup>	REF	DR CALUX		HRGC/HRMS (AL/ML+mu)				
Matrix	pg TEQ/ g fat	pg TEQ/ g fat	tested	suspected > REF	Samples >REF	Samples >AL <sup>2,5</sup>	Samples >ML <sup>3,5</sup>	Neg's tested	>AL
Pork	0.6/0.5	0.5	94	0	0	0 (0/0)		16	0
Poultry	1.5/1.5	0.9	54	0	0	0 (0/0)		15	0
Bovine	1.5/1.0	0.9	87	10	8	5 (0/5)		17	0
Sheep	1.5/1.0	0.9	79	36	35	11 (6/10)	1 (0/1)	7	0
Deer <sup>4</sup>	1.5/1.5	0.9	6	6	6	5 (3/5)	4 (3/4)	0	na
Eggs	2.0/2.0	1.9	106	17	4	0 (0/0)		22	0
Milk	2.0/2.0	1.9	78	3	2	0 (0/0)		21	0
Total			504	72	55	21 (9/20)	5 (3/5)	98	0

1. ALs for dioxins/dl-PCBs,
2. Samples exceeding one or both ALs (samples exceeding ALs for dioxins/dl-PCBs),
3. Samples exceeding one or both MLs (samples exceeding MLs for dioxins/sum),
4. No official limit for deer; for comparison the limits for game were used,
5. evaluation against AL and ML included 20% measurement uncertainty



# Evaluation of dataset



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# Conclusions

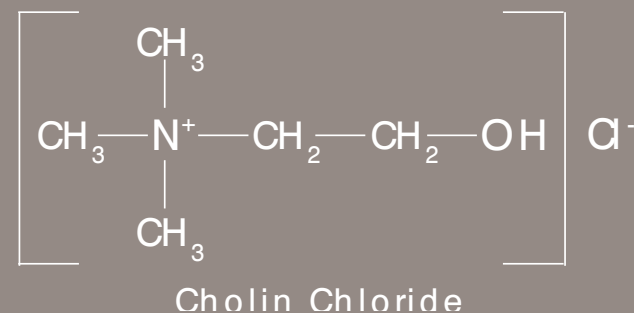
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- CALUX assay is a valuable tool for screening, especially for routine monitoring where most samples will be negative
- Suspected samples should be analyzed by GC/HRMS analysis
  - Confirmation of dioxins/dl-PCBs
  - Determination of TEQ-level
  - Determination of congener pattern: source identification
- Combined use of a bioassay and a confirmation method best strategy for detecting novel risks

# Detection of novel risks?

# Cholin Chloride

- Feed additive (up to 1 g/kg)
- Positive test response in DR CALUX (different samples)
- Indicative level around 5 ng BEQ/kg
- GC/HRMS: dioxins and dioxin-like PCBs below LOQ
- Identity confirmed by NMR: no mix-up

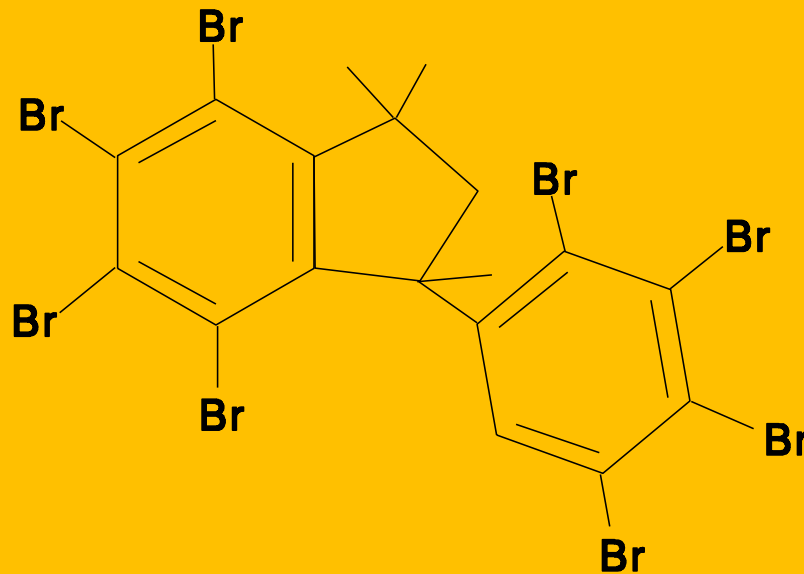


# GC-TOF analysis

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- Sample contaminated by brominated compounds
- PBDEs and tribromophenol
- Unknown compound with 8 bromo-groups and molecular weight of 867
  - Found through internet: FR-1808
  - Ordered and confirmed

# FR-1808 (OBIND)



octabromo-1,3,3-trimethyl-1-phenylindan  
FR-1808 (Mw 867.52)

Negative in  
DR CALUX

# Brominated compounds

samples 208908 and 210099)

- PBDEs

- 47: 4 and 78 ng/kg
- 49: 0.5 and 131 ng/kg
- 99: 2 and 150 ng/kg
- 100: 0.4 and 17 ng/kg

- FR-1808: 140 and 700 ng/kg (CALUX neg)

- 2,4,6-tribromophenol: 1100 and 3600 ng/kg

- And ..... brominated dioxins

- Considered equally toxic as chlorinated dioxins



# Other application fields

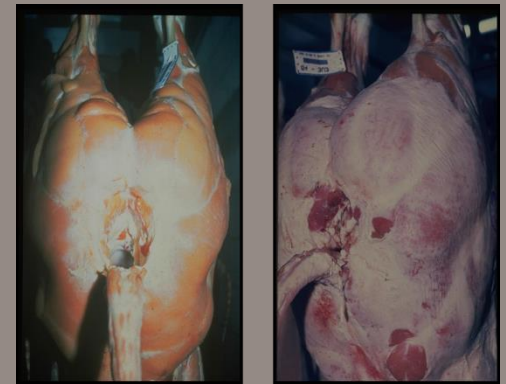


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# Hormonal substances

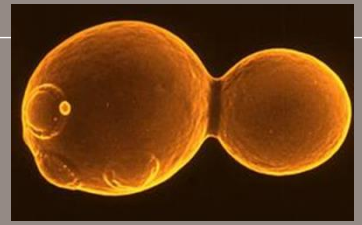
- Interesting area for fraud: increased growth and better feed conversion
- Yeast assays for oestrogens, androgens, corticosteroids
- Receptor assay for beta-agonists
- Tests validated and run under accreditation
  - for feed and urine
- Decision limit based on negative samples
  - Any sample with abnormal response is examined



# Adulterants in food supplements

- Addition of pharmacologically active substances
  - To make it work
- Examples
  - Diethylstilboestrol in Prostatol
  - Androgens in anabolic supplements
  - Beta-agonists in fat burners
  - Sibutramine in slimming products
  - Viagra-like compounds in potency products

# Food supplements



- Dietary supplements → analysed by LC-MS/MS for 49 steroids.

- 18 supplements - 11 positive and 7 negative

all positive in the yeast androgen assay (RAA)

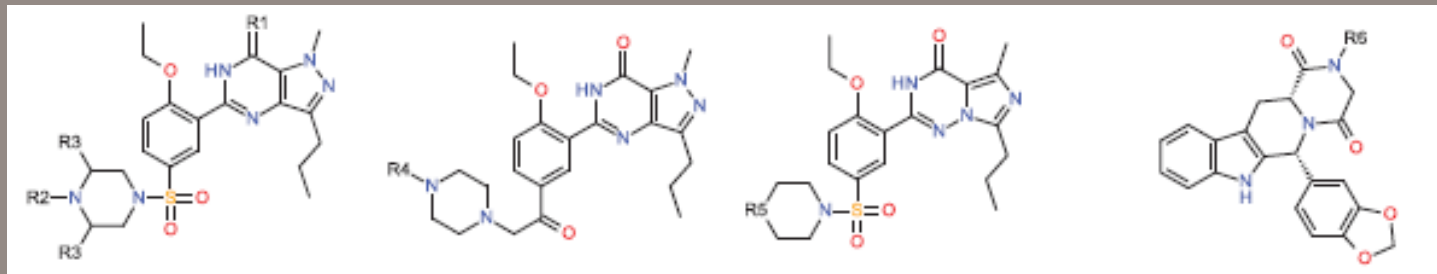
2 supplements showed androgenic activity in the yeast androgen assay (RAA):  
additional steroids in both confirmed (1-testosterone)



# Viagra-like compounds in natural products

## PDE-5 (phosphodiesterase) inhibitors

- Horny goat weed (yin yang ho)
  - Contains icariin
  - Weak PDE-5 inhibitor
- In most cases sildenafil,
  - or other synthetic analogues



# Dose response curves

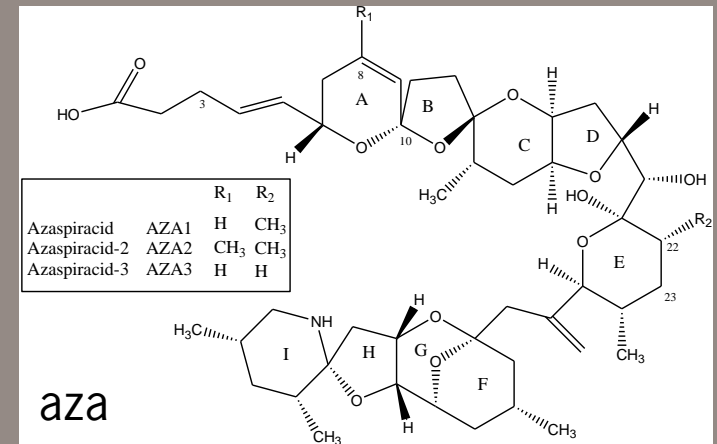
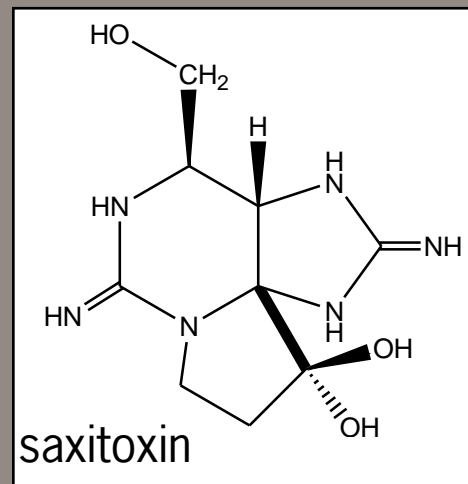
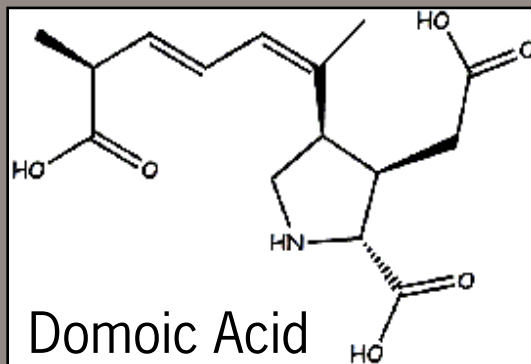
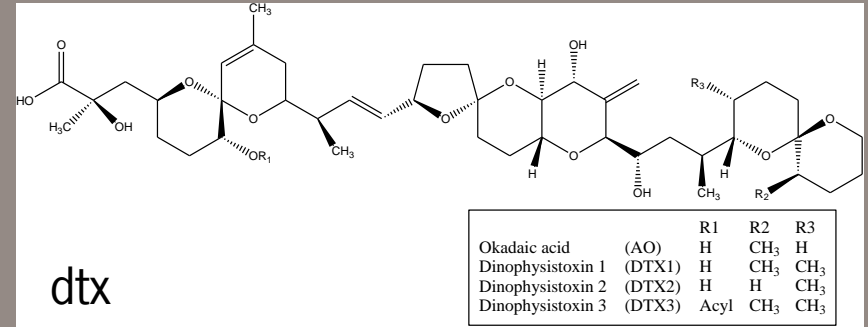
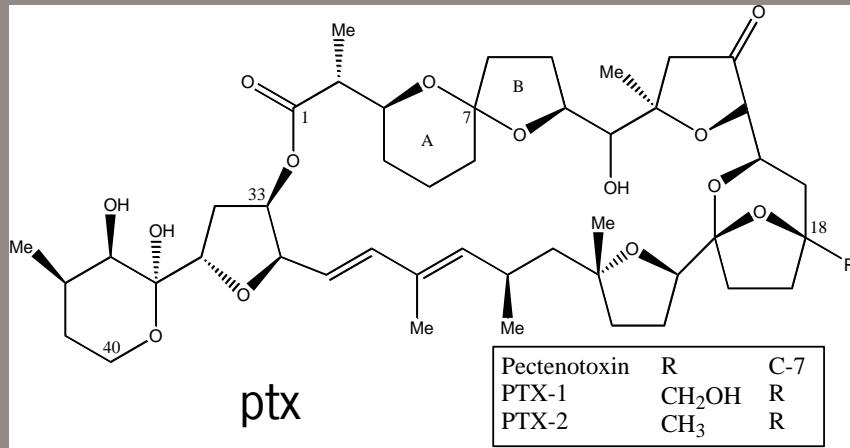
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# Food supplements

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# Marine biotoxins: PSPs, DSPs, ASPs



# EU legislation



- Regulation 2074/2005/EC
  - Mouse bioassay (MBA) reference method
  - Rat bioassay has been used for DSPs

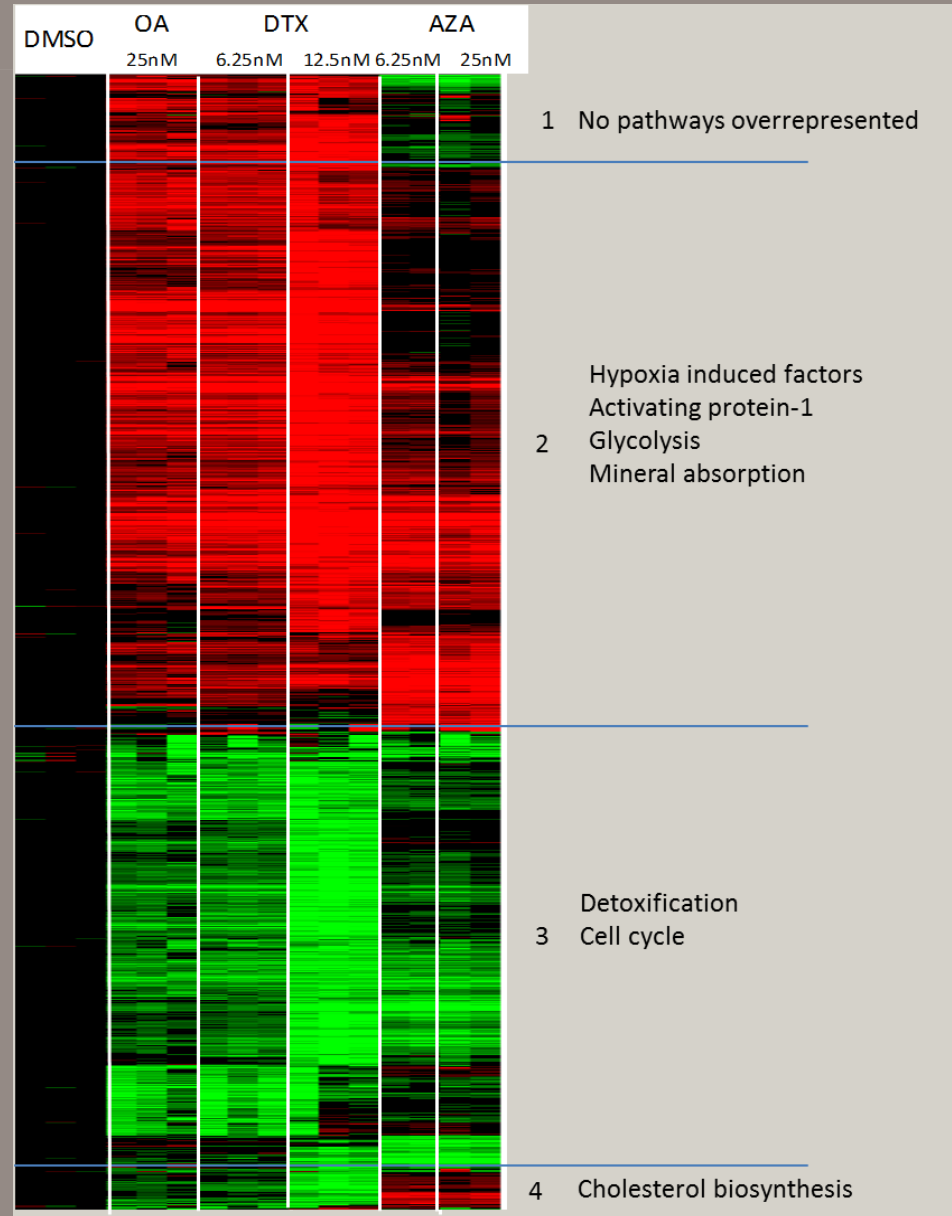


- Regulation 15/2011/EC
  - LC-MS/MS reference method for DSPs
  - For PSPs mouse bioassay, or Lawrence method (LC/fluorescence)
  - For harvest areas MBA still allowed (novel risks)
  - Lack of standards for all toxins
- Alternatives needed



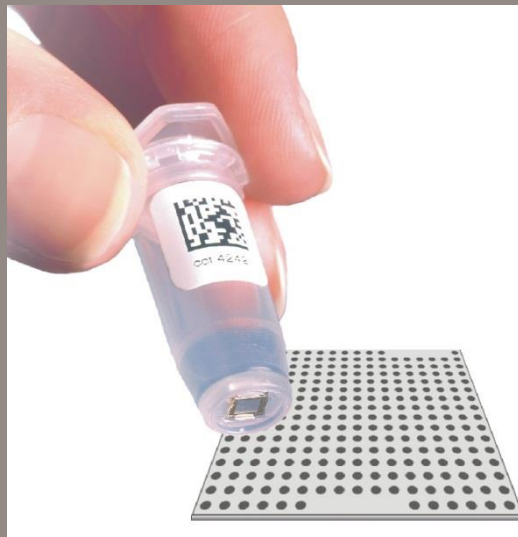
# Gene expression?

- CaCo-2 cells exposed to OA, DTX and AZA
- Full genome array
- Various genes selected



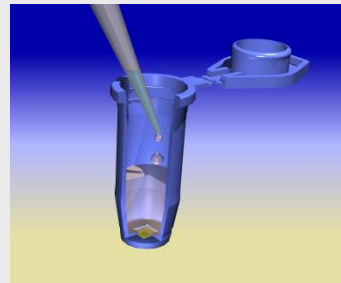
# Array Tube for marine toxins (EU Biocop)

## Transcriptomics assay on Clondia **AT-Platform** >> **ArrayTube (AT) Platform**



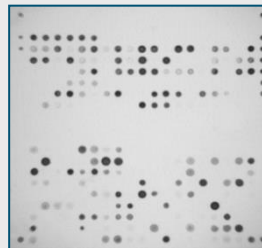
single tube format  
based on conventional  
laboratory vials  
(Clondia, nowadays Alere)

### microtube

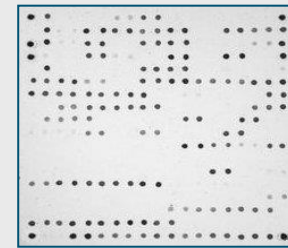


- easy processing with standard lab equipment
- no evaporation
- uniform wettability
- small volumes
- optimal processing through small surface area

### + microarray



*protein (HLA) array*



*oligonucleotide array*

- custom manufacturing of protein/peptide or nucleic acid based arrays
- array size of 2mm x 2mm with up to 300 features
- arrays including reaction control spots



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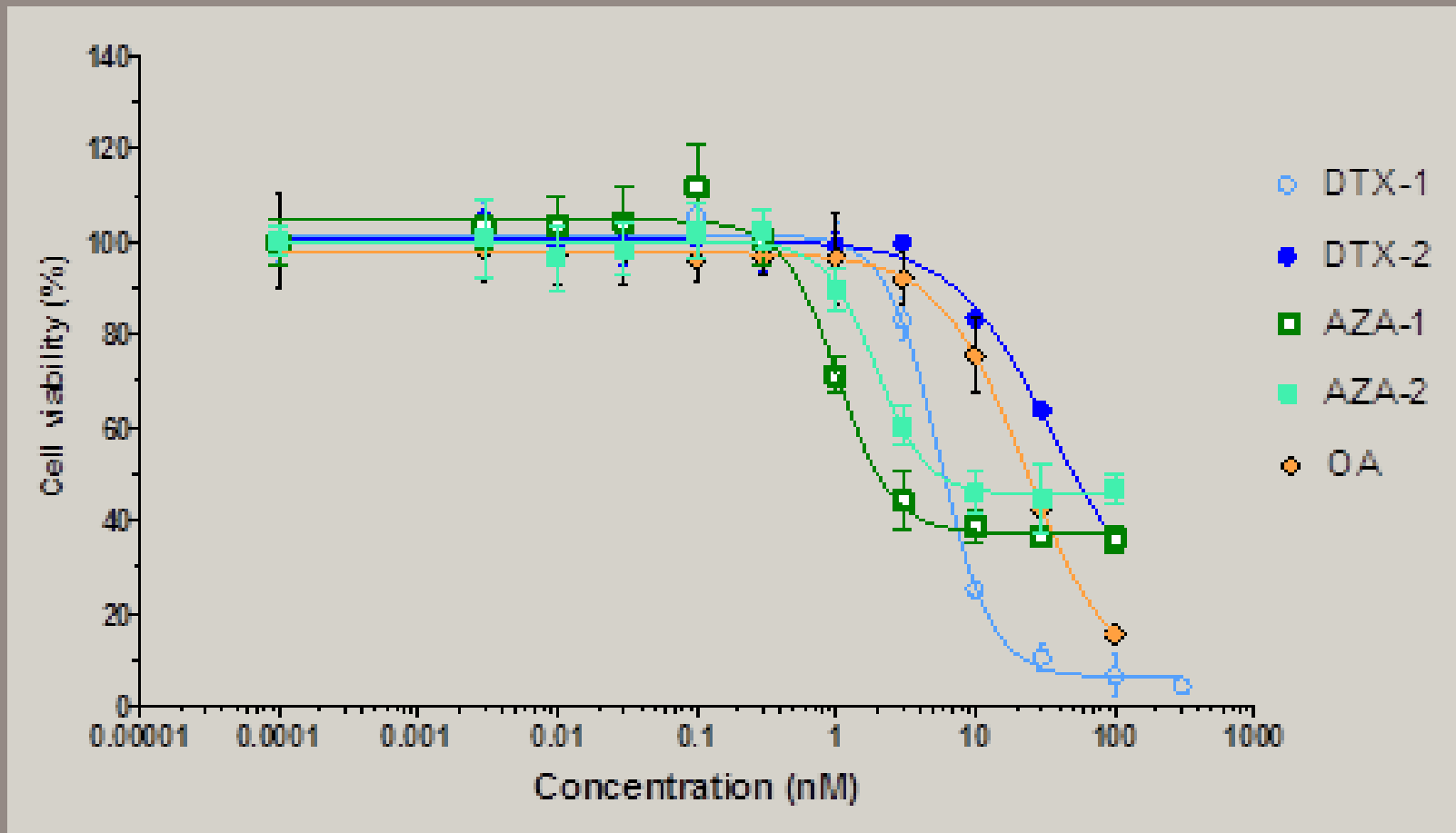
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# Luminex

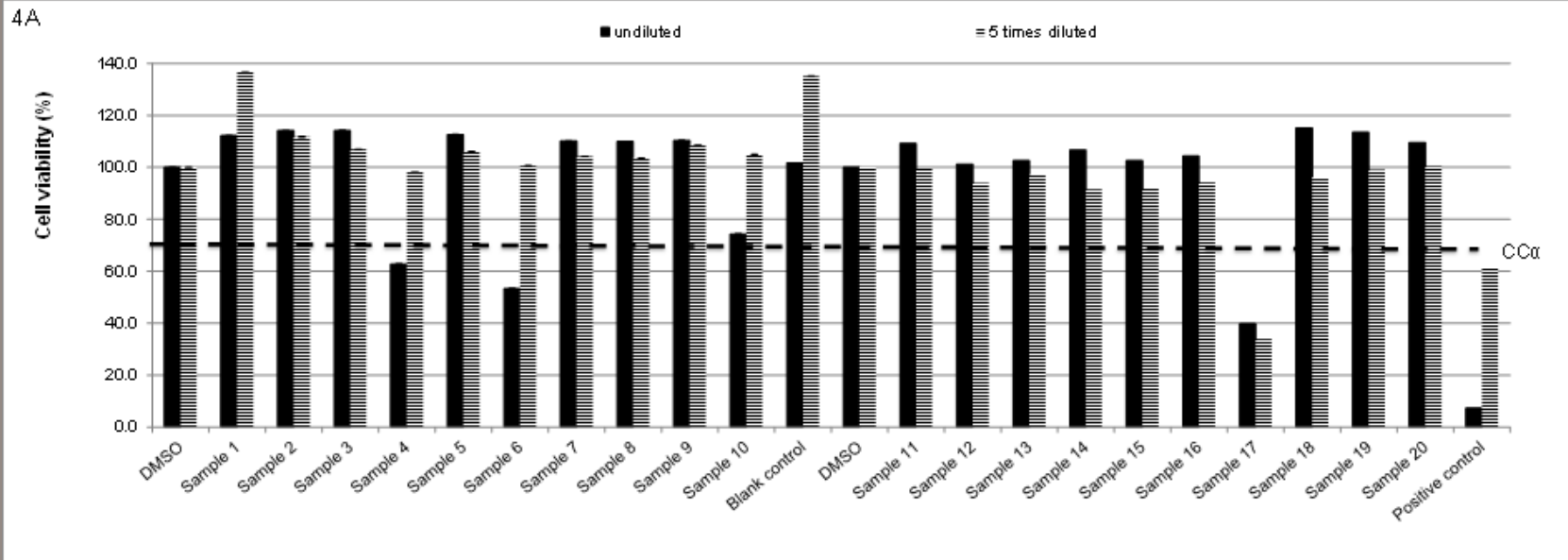
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# Neuro-2a assay (reduced MTT reduction)



# Shellfish samples from Chile

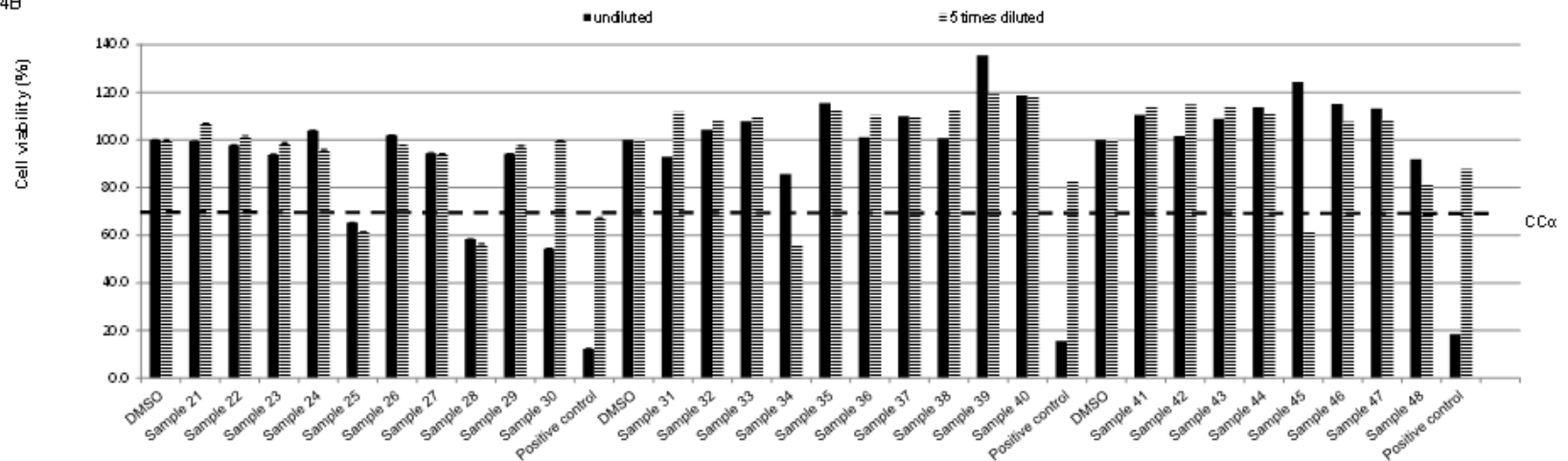
Primarily yessotoxins



# Shellfish samples from Chile

Primarily yessotoxins

4B



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# Also works for PSPs and NSPs

- Saxitoxins
- Ciguatera toxins (much more sensitive than LC/MS)
- Tetrodotoxins

# Questions?



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