# Dioxins and PCBs: incidents with feed and food

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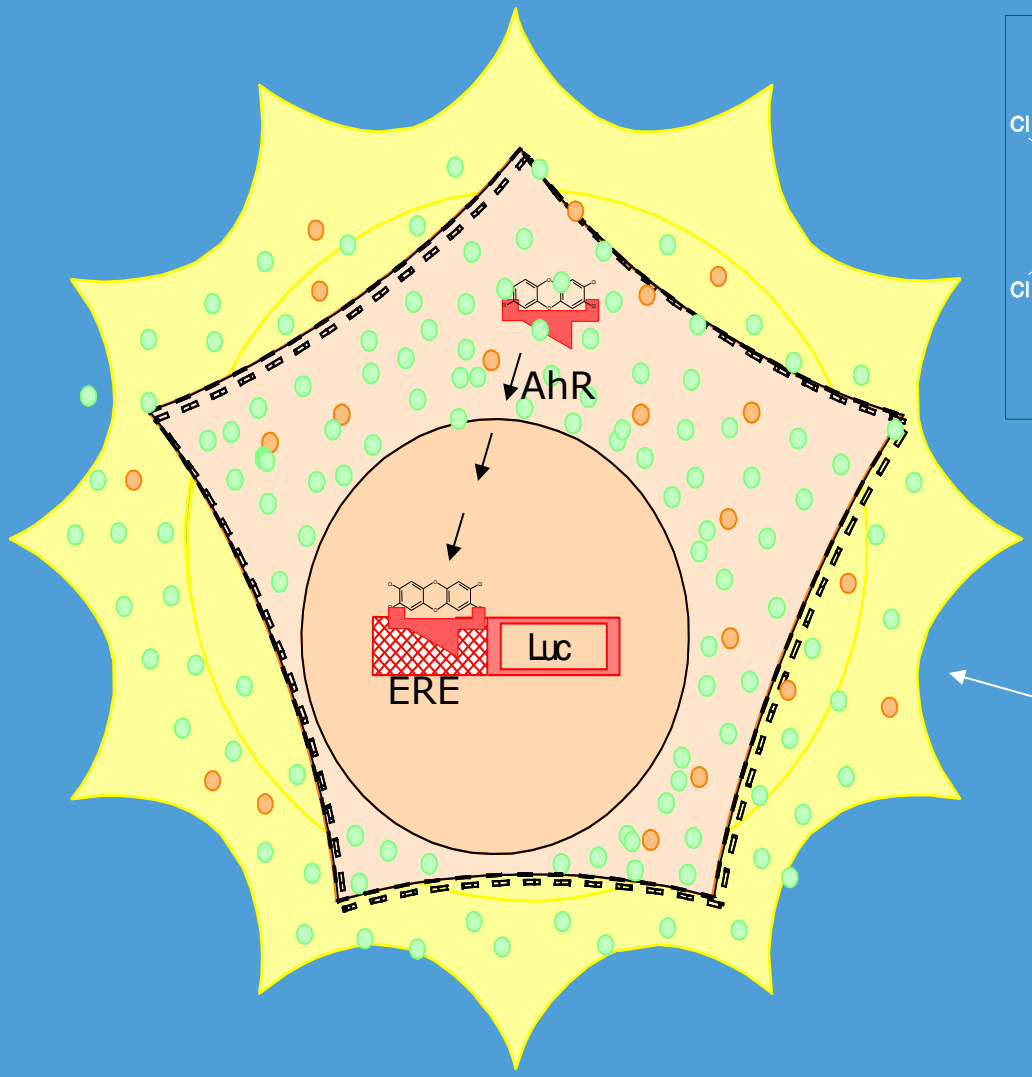
# **Bioassays**: effect based screening assays for the detection of hormones and EDCs

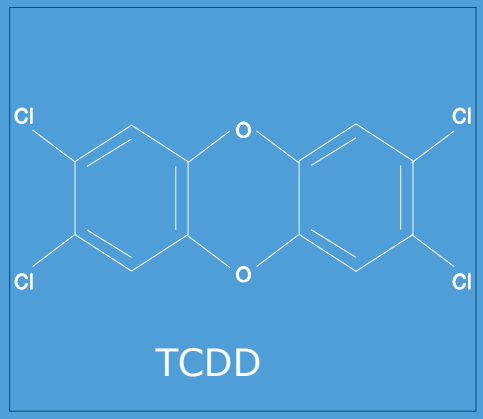
- THIS PRESENTATION: the DR-CALUX®, a bioassay based on a rat liver cell line (H4IIE) for the detection of dioxins and dioxin-like PCBs

# History bioassays at RIKILT-Institute of Food Safety, Wageningen University and Research Centre

- Started a long time ago
  - Sensory lab - food test panel (in vivo)
  - Histology (in vivo)
  - Inhibition assays for the detection of antibiotics
  - Toxicological studies using cells (cell lines, freshly isolated pig liver cells)
  - DR-CALUX® for the detection of dioxins and DL-PCBs

# DR-CALUX® for dioxins




Luciferin
+
ATP

Aarts et al. 1993

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# DR-CALUX® for dioxins

- Validated for feed, additives, animal fat and plant oils
- Heavily used during many (big) incidents
  - Brazilian citrus pulp 1998
  - Belgian PCB fat 1999
  - Belgian choline chloride 2002
  - German bakery waste 2003
  - Potato peels/kaolinic clay 2004
  - Feed fat Netherlands (gelatin fat from Belgium) 2006
  - Minerals (Zinc) Chile 2008
  - Bakery waste Ireland 2008
  - Ukraine maize 2010
  - German bio-fuel oils in feed 2010-2011

# 1 Feed fat Netherlands (gelatin fat from Belgium) 2006

- Extended survey on fats and other ingredients reused for animal feed
- Fat positive in the DR-CALUX®
- Confirmed by the HRGC/HRMS reference method (50 pg TEQ/g)
- Even two higher samples of fat: 220 and 440 pg TEQ/g
- 1.5 to 5% used for feed: theoretically 22 ng TEQ/kg
- Fatty acid pattern >>> pig fat

# 1 Feed fat Netherlands (gelatin fat from Belgium) 2006

Table 1. Result of the screening of the first fat sample (number 200163193; 50 ng TEQ/kg) with the DR-CALUX® bioassay, showing a clearly elevated response. At RIKILT the response of the sample extract, tested in triplicate, is compared with that of a set of reference samples.

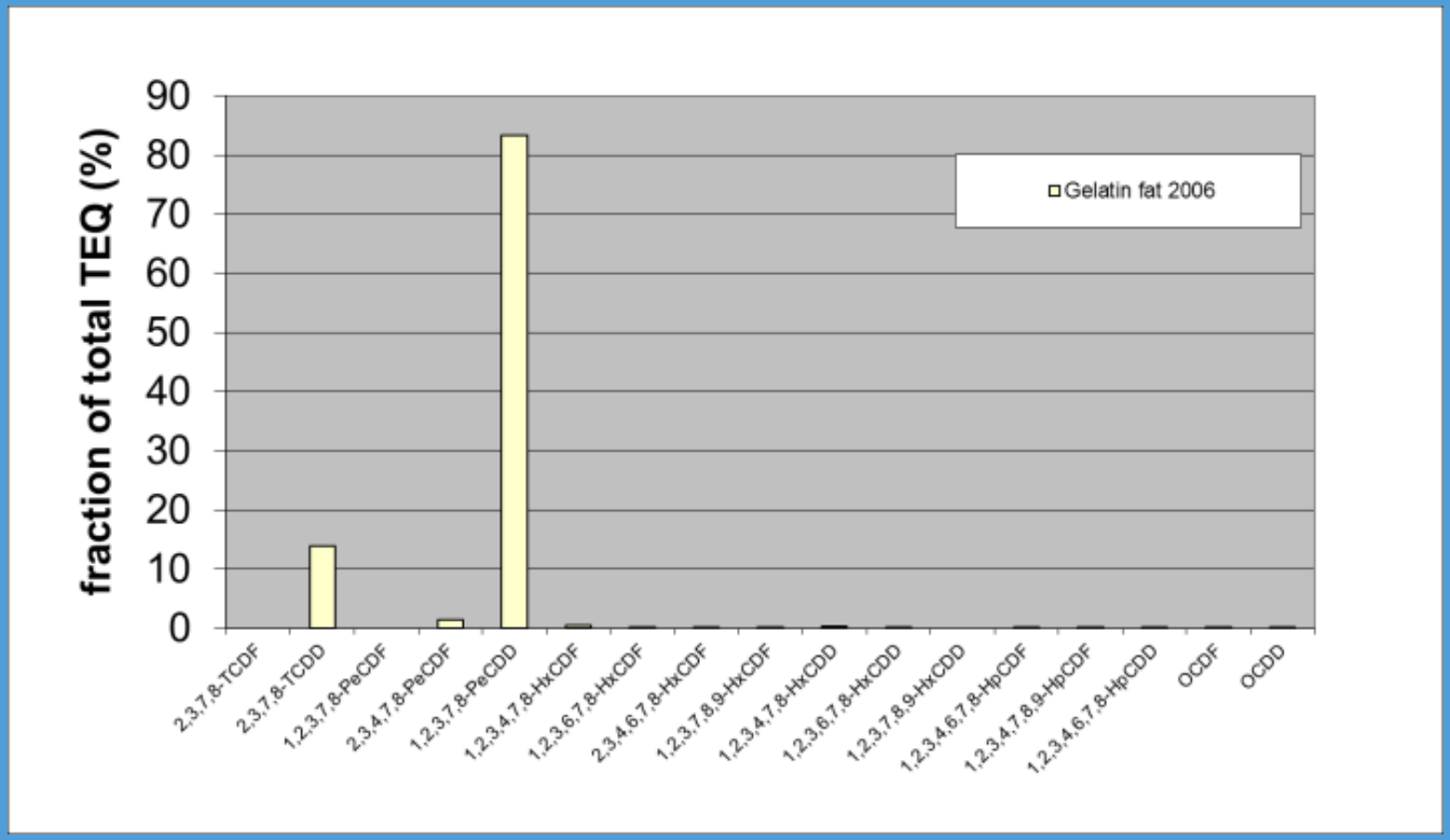
| RIKILT number | Product | Signal 1 (RLUs) | Signal 2 (RLUs) | Signal 3 (RLUs) | Mean | SD | Difference | Decision |
|---|---|---|---|---|---|---|---|---|
| Blank fat | | 52 | 74 | 75 | 67 | 13 | 0 | |
| Reference 1 | 0.38 pg TEQ/g | 74 | 73 | 78 | 75 | 2 | 8 | |
| Reference 2 | 0.75 pg TEQ/g | 107 | 104 | 95 | 102 | 6 | 35 | |
| Reference 3 | 1.5 pg TEQ/g | 182 | 161 | 160 | 168 | 13 | 101 | |
| Reference 4 | 3.0 pg TEQ/g | 214 | 286 | 237 | 246 | 37 | 179 | |
| 200163193 | Animal fat | 888 | 1030 | 905 | 941 | 77 | 874 | Suspected |

Hoogenboom et al., 2006, Organohal. Comp. 68, 193-196

# 1 Feed fat Netherlands (gelatin fat from Belgium) 2006

- Pattern: only 2,3,4,7-TCDD and 1,2,3,7,8-PeCDD and almost no non-2,3,7,8 substituted dioxin congeners.
- Pattern not recognized from the literature or by any expert in the field.
- No useful info coming from additional studies with GC/TOF-MS (some chlorinated phenols and several PAHs)
- Tracking and tracing showed the fat was delivered to a Dutch feed company by a Belgium fat recycler
- The hydrochloric acid (HCl) used for dissolving the pig bones for gelatin fat production is thought to be the source, but still not sure

# 1 Feed fat Netherlands (gelatin fat from Belgium) 2006




Hoogenboom et al., 2006, Organohal. Comp. 68, 193-196

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# 1 Feed fat Netherlands (gelatin fat from Belgium) 2006

| DR-CALUX® / GC/MS | Negative samples (n=80) | | | Suspected samples (n=78) | | |
|---|---|---|---|---|---|---|
| | True negative | False negative type 1 | False negative type 2 | False Positive | True positive type 1 | True positive type 2 |
| | 75 | 5 | **0** | **16** | 26 | 36 |

Type 1 based on the action limits
Type 2 based on the tolerance limits

- Overall 218 samples tested: (feed, animal fat for feed, pig fat and chicken fat), 78 suspect and 140 negative
- The 78 suspect and 80 of the negative samples were analysed by HRGC/HRMS >>> **no false negatives and only 16 false positives**

Hoogenboom et al., 2006, Organohal. Comp. 68, 193-196

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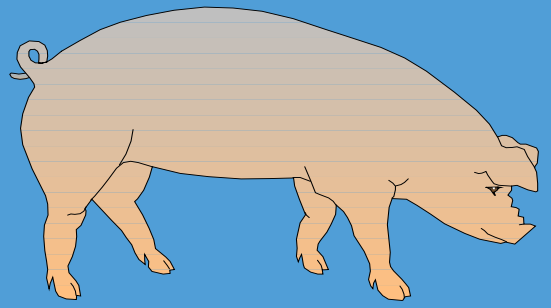
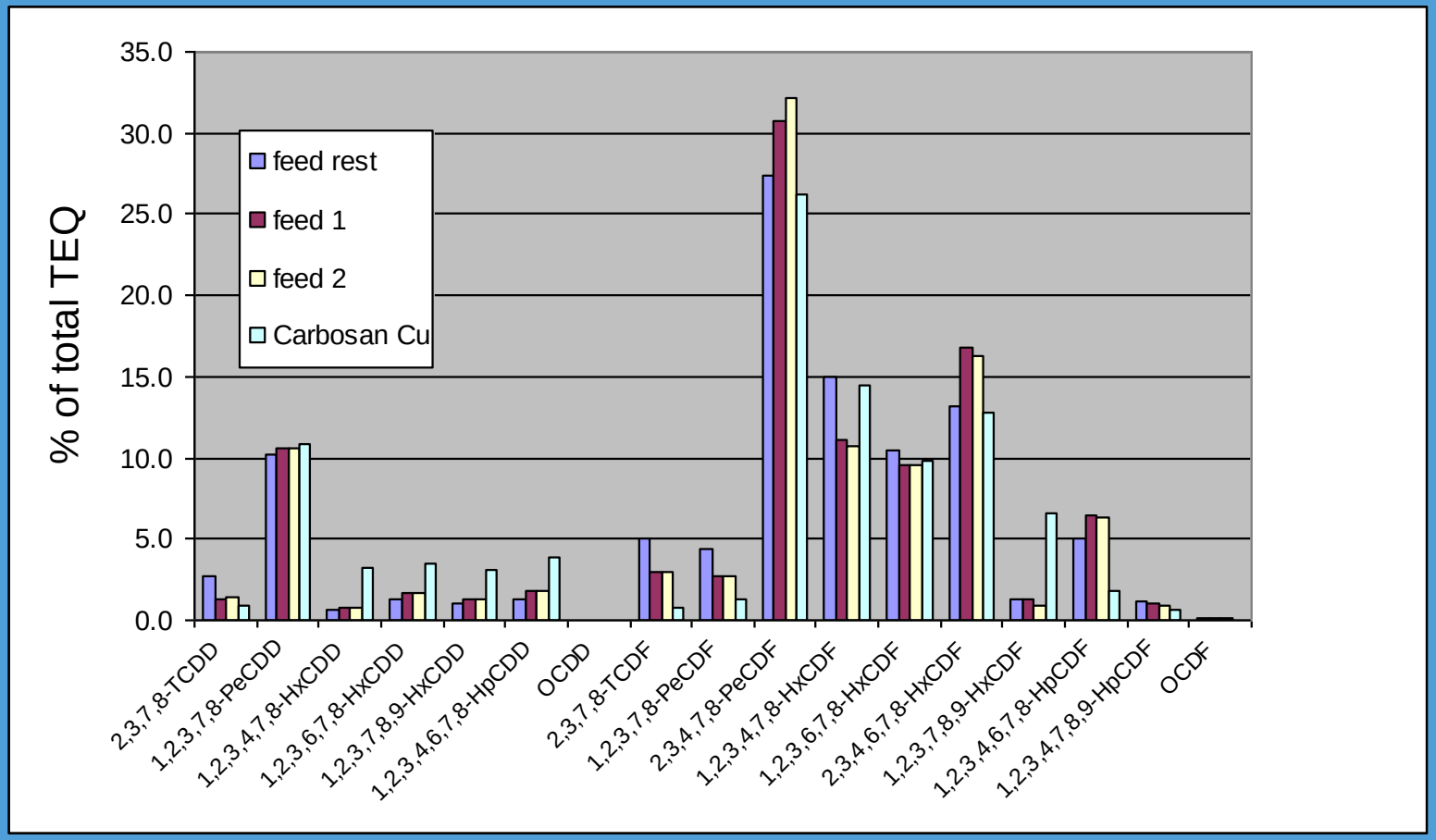
# 2 Incident in Chile 2008



- Discovered in South-Korea
- Dioxins in pig meat from Chile
  - Borders closed in Korea en Japan (>4 months)
  - Later, also positives in Greece
- Samples of pig fat contained around 10-20 pg TEQ/g
  - normal <<1 pg TEQ/g fat
- Two feed samples contained around 14 ng TEQ/kg

# 2 Incident in Chile 2008




Pattern points to contaminated mineral mix

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# Current application

- Not compared to a sample spiked on the action limit, but calculated from a fitted dose-response curve obtained with spiked reference samples
- Calculated amount compared to the action limit

# Current application

| RIKILTnummer | Product | totaal TEQ pg TEQ/g | Opdrachtgever | Opdrachtgeversnr. | volg. nr. | Inweeg (g) | signaal 1 | signaal 2 | signaal 3 | signaal mean | signaal SD | Gehalte ng BEQ/kg | Resultaat | Problemen | Rapport | %CV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BV0,5 | Ned. botervet | 0.52 | | | 1 | 2.012 | 143.46 | 132.67 | 138.36 | 138 | 5 | 0.53 | oke | | | 3.9 |
| BV1 | Ned. botervet | 0.97 | | | 2 | 2.003 | 216.3 | 200.65 | 182.63 | 200 | 17 | 0.99 | oke | | | 8.4 |
| BV2 | Ned. botervet | 1.87 | | | 3 | 2.003 | 299.25 | 294.25 | 287.19 | 294 | 6 | 1.74 | oke | | | 2.1 |
| BV3 | Ned. botervet | 2.8 | | | 4 | 1.995 | 322.62 | 489.83 | 454.83 | 422 | 88 | 2.92 | oke | | | 20.9 |
| BV6 | Ned. botervet | 5.62 | | | 5 | 2.004 | 724.34 | 663.59 | 565.43 | 651 | 80 | 5.60 | oke | | | 12.3 |
| 311499 | Varken | | NP | | 6 | 2.001 | 72.49 | 71.5 | 61.39 | 68 | 6 | 0.05 | negatief | | ja | 9.0 |
| 311500 | Rund | | NP | | 7 | 2.002 | 134.29 | 131.67 | 128.2 | 131 | 3 | 0.49 | negatief | | ja | 2.3 |
| 311501 | Rund | | NP | | 8 | 2.004 | 163.53 | 145.6 | 159.6 | 156 | 9 | 0.67 | negatief | | ja | 6.0 |
| 311502 | Varken | | NP | | 9 | 1.997 | 67.78 | 66.53 | 67.98 | 67 | 1 | 0.04 | negatief | | ja | 1.2 |
| 311503 | Vleeskuiken | | NP | | 10 | 1.997 | 81.98 | 78.04 | 75.19 | 78 | 3 | 0.12 | negatief | | ja | 4.3 |
| 311504 | Vleeskuiken | | NP | | 11 | 1.998 | 64.48 | 58.97 | 49.39 | 58 | 8 | -0.02 | negatief | | ja | 13.3 |
| 311505 | ov Pluimvee | | NP | | 12 | 2.002 | 85.16 | 90.45 | 86.84 | 87 | 3 | 0.18 | negatief | | ja | 3.1 |
| 311506 | Vleeskuiken | | NP | | 13 | 2.001 | 91.94 | 90.31 | 81.8 | 88 | 5 | 0.18 | negatief | | ja | 6.2 |
| 311507 | Vleeskuiken | | NP | | 14 | 2.008 | 89.38 | 87.19 | 71.05 | 83 | 10 | 0.15 | negatief | | ja | 12.1 |
| 311508 | Vleeskuiken | | NP | | 15 | 1.998 | 70.85 | 69.53 | 41.32 | 61 | 17 | 0.00 | negatief | | ja | 27.5 |
| 311509 | Vleeskuiken | | NP | | 16 | 2.012 | 77.23 | 64.44 | 65.05 | 69 | 7 | 0.05 | negatief | | ja | 10.5 |
| 311510 | Rund | | NP | | 17 | 1.993 | 143.93 | 140.14 | 103.71 | 129 | 22 | 0.47 | negatief | | ja | 17.2 |
| 311511 | Varken | | NP | | 18 | 1.996 | 51.95 | 57.69 | 33.12 | 48 | 13 | -0.09 | negatief | | ja | 27.0 |
| 311512 | Mestkalf | | NP | | 19 | 2.006 | 82.57 | 76.55 | 88.83 | 83 | 6 | 0.15 | negatief | | ja | 7.4 |
| 311819 | Vleeskuiken | | NP | | 20 | 2.009 | 47.21 | 50.24 | 52.18 | 50 | 3 | -0.07 | negatief | | ja | 5.0 |
| 311820 | Vleeskuiken | | NP | | 21 | 1.996 | 42.25 | 46.74 | 43.28 | 44 | 2 | -0.11 | negatief | | ja | 5.3 |
| | | | | | | | | | | | | | | | | 9.8 |

Exponential
y=a0+a1*exp(-x/a2)

| r2 Coef Det | DF Adj r2 | Fit Std Err | F-Statistic |
|---|---|---|---|
| 0.997842 | 0.995684 | 13.382297 | 462.353111 |

| | Value | Std Error | t-Value | 95% Confidence Limits |
|---|---|---|---|---|
| a0 | 1181.33 | 248.02 | 4.76 | 114.0823012248.57142 7 |
| a1 | -1120.37 | 234.21 | -4.78 | -2240.74 |
| a2 | 7.49 | 2.47 | 3.03 | -3.15783218.128612 |

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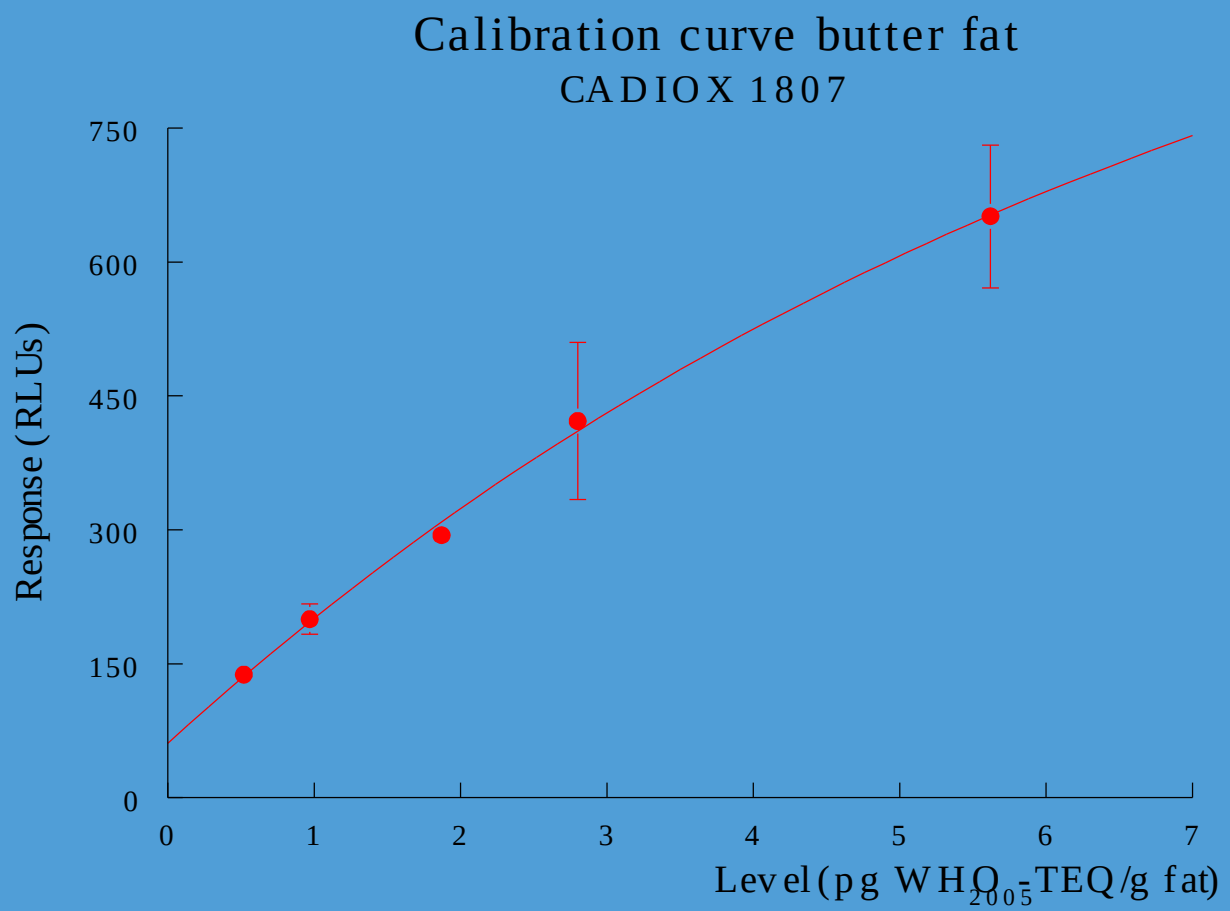
# Current application

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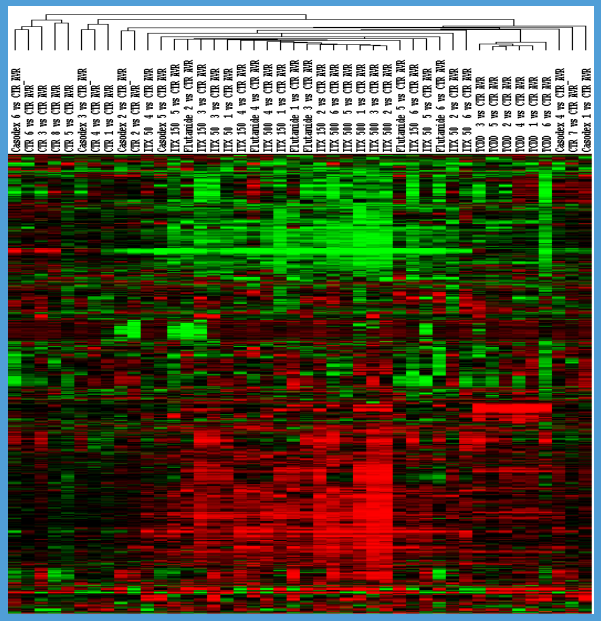
The added value: an additional example were bioassays are used and results are compared with omics based methods and in vivo outcomes

ITX



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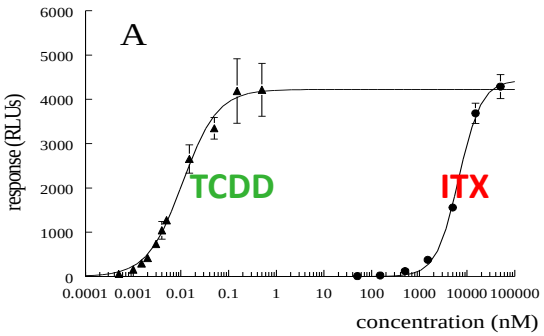
# Regarding ITX

- 2-Isopropylthioxanthone (2-ITX) is a photo initiator in printing ink of packaging materials (e.g. found in milk).
- Little is known about possible effects of ITX on human health.
- Here we test the *in vitro* AhR agonist activity and both the (anti-)estrogenic and (anti-)androgenic properties of ITX with bioassays first.

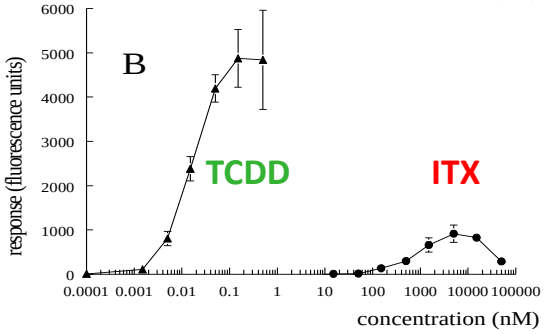
# ITX in the DR-CALUX® and yeast estrogen and androgen bioassays

## Rat liver H4IIE cells

CALUX

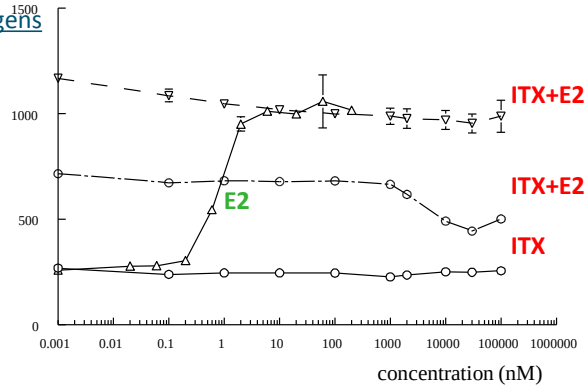



EROD

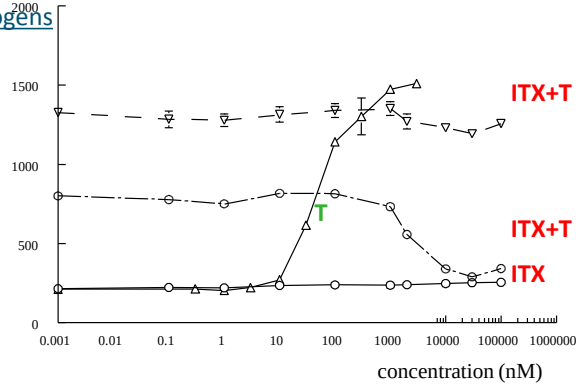



## Yeast cells

Estrogens




Androgens

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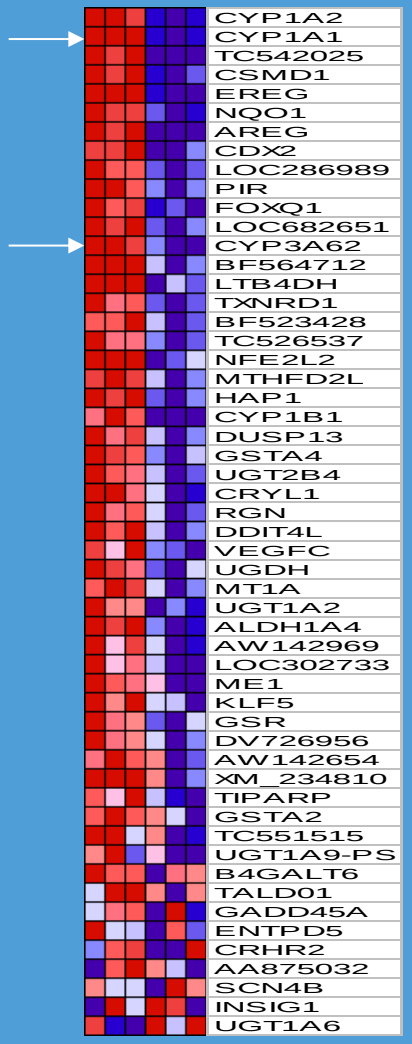
Peijnenburg et al., *TiV* **24** (2010) 1619-1628

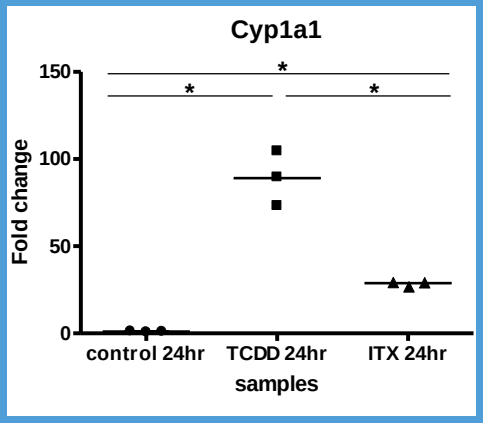
# *In vitro* gene expression profiles in H4IIE cells: ITX vs TCDD

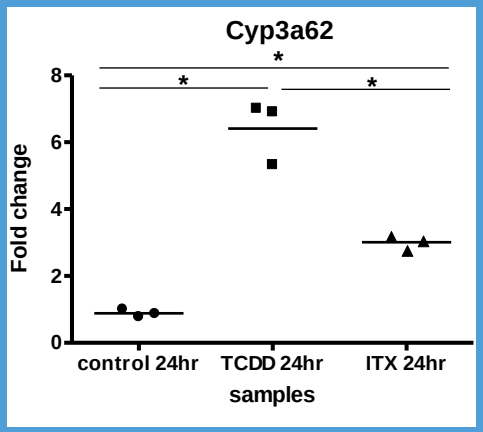
ITX/DMSO




Genes differentially expressed by TCDD were selected on an average up-regulation in rat H4IIE hepatoma cells by ≥1.5-fold at 24 h in combination with a P-value of <0.01 (students t-test). The heat map shows the relative expression levels of these TCDD-affected genes in hepatoma cells treated for 24 h either with 5 µM ITX or with the vehicle DMSO (CTR). Red: relatively high expression; blue: relatively low expression.

⇒ The vast majority of TCDD-up-regulated genes are also up-regulated by ITX.




RT-PCR confirmation of microarray data. Quantitative real-time PCR was performed for Cyp1a1 and Cyp3a62. The expression levels of the genes are relative to tubulin alpha 1C (Tuba1c). The mean gene expression value within each group is indicated with —. Significant difference between the groups: *P < 0.005, **P < 0.05.

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Peijnenburg et al., *TiV* **24** (2010) 1619-1628

# Male puberty test: summary of all *in vivo* results

| | Body weight | Preputial separation | Sem vesicles + Coag gland | Ventral prostate | Testes | Epididymi | Liver | Kidneys | Sperm count caput | Sperm count cauda | Sperm motility |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ITX-50 | | | | ↓ | | | ↑ | | | | |
| ITX-150 | | | ↓ | ↓ | | | ↑↑ | | | | |
| ITX-500 | ↓ | | ↓↓ | ↓↓ | | | ↑↑↑ | | | ↓ | |
| TCDD | | | | | | | | | | | |
| Flutamide | ↓ | ↑↑↑ | ↓↓↓ | | | | | | ↓↓↓ | ↓↓↓ | ↓↓↓ |
| Casodex | | ↑↑↑ | ↓↓↓ | | | | | | ↓↓ | ↓↓ | ↓↓ |

**Anti-androgen** **R-activity** **Anti-androgenic**

Flutamide & Casodex only

Flutamide & Casodex only

Flutamide & Casodex only

Flutamide & Casodex only

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Hendriksen et al., in preparation

# Conclusions

- Validated DR-CALUX® bioassay for dioxins that can be used in routine monitoring programs.
- Intensively used during several incidents over the past 20 years and able to reduce MS-workload (negatives).
- Added value shown, i.e. the detection and identification of unknown contaminants.
- Identification of the source of the contamination, always successful, except once in the Belgian gelatine fat incident?

# Questions?

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