Development of effect based screening assays for the detection of marine toxins, using a genomics approach

October 18th, BfR Berlin

Toine Bovee, Peter Hendriksen, Liza Portier, Ron Hoogenboom

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Why effect based assays?
EU Regulations I

- Because we had good experience with the DR-CALUX screening for dioxins and PCBs (Friday Dr. Hädrich)
- **We began with the hormones:** Directive 96/23/EC: banns the use of Group A substances (list of compounds: targeted analytical analysis),
    - Stilbenes, derivatives, salts and esters
    - Antithyreogene compounds
    - Steroids
    - Resorcyclic Acid Lactones (including zeranol)
    - ß-agonists
    - Others, as mentioned in the Annex of Regulation EC 37/2010

However,....
EU regulations II

- Directive 96/23/EC mentions hormonal action and refers to Directive 96/22/EC
- Directive 96/22/EC: Prohibits all substances having hormonal action
- Regulations EC 178/2002 and EC 882/2004: oblige the member states to identify emerging risks and use validated and accredited methods for control analysis

How to obey to all these laws ?

- The only way is bioactivity screening combined with chemical analytical confirmation and identification using validated and accredited methods for both
- Or...to get rid of the laws. But would that be safe?
- ?

Bioactivity measurements for hormones

Transcriptional Activation (TA) bioassays (yeast or mammalian cell based)

- Detect all compounds (structures) that are able to activate the receptor, e.g. the estrogen, androgen, progesterone or glucocorticoid receptor. As the main mode of action of these hormones is by activating their receptor, these TA bioassays fulfil Directive 96/22/EC that prohibits all substances having hormonal action, and they are also able to detect new designer steroids and new risks.

- Moreover, they are:
  - Sensitive and specific
  - Quick, simple and robust
  - Applicable to urine, feed and preparations

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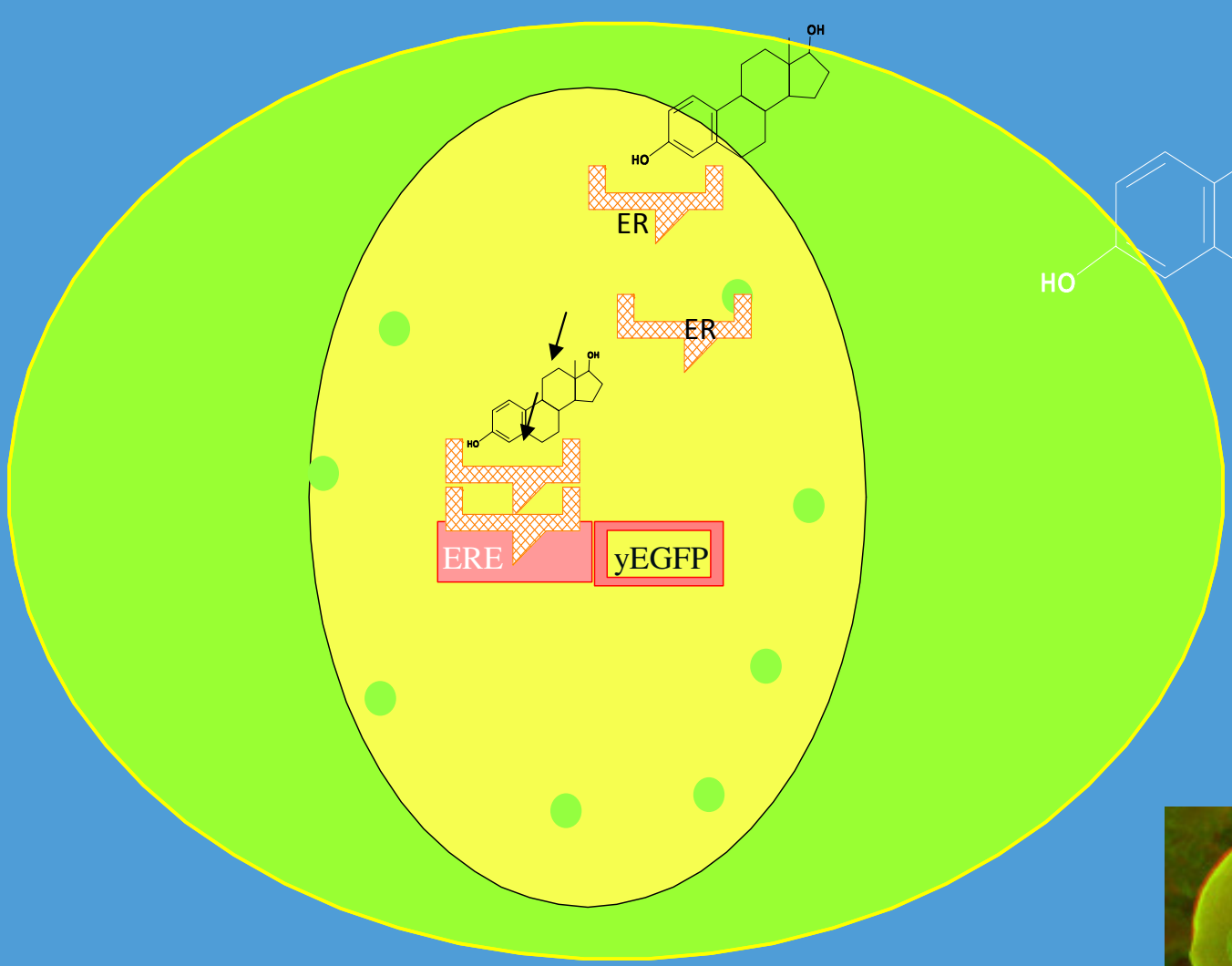
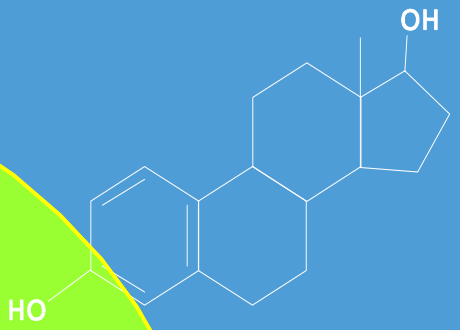
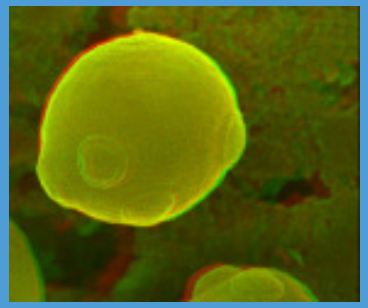
Development of a yeast estrogen bioassay





17β-estradiol



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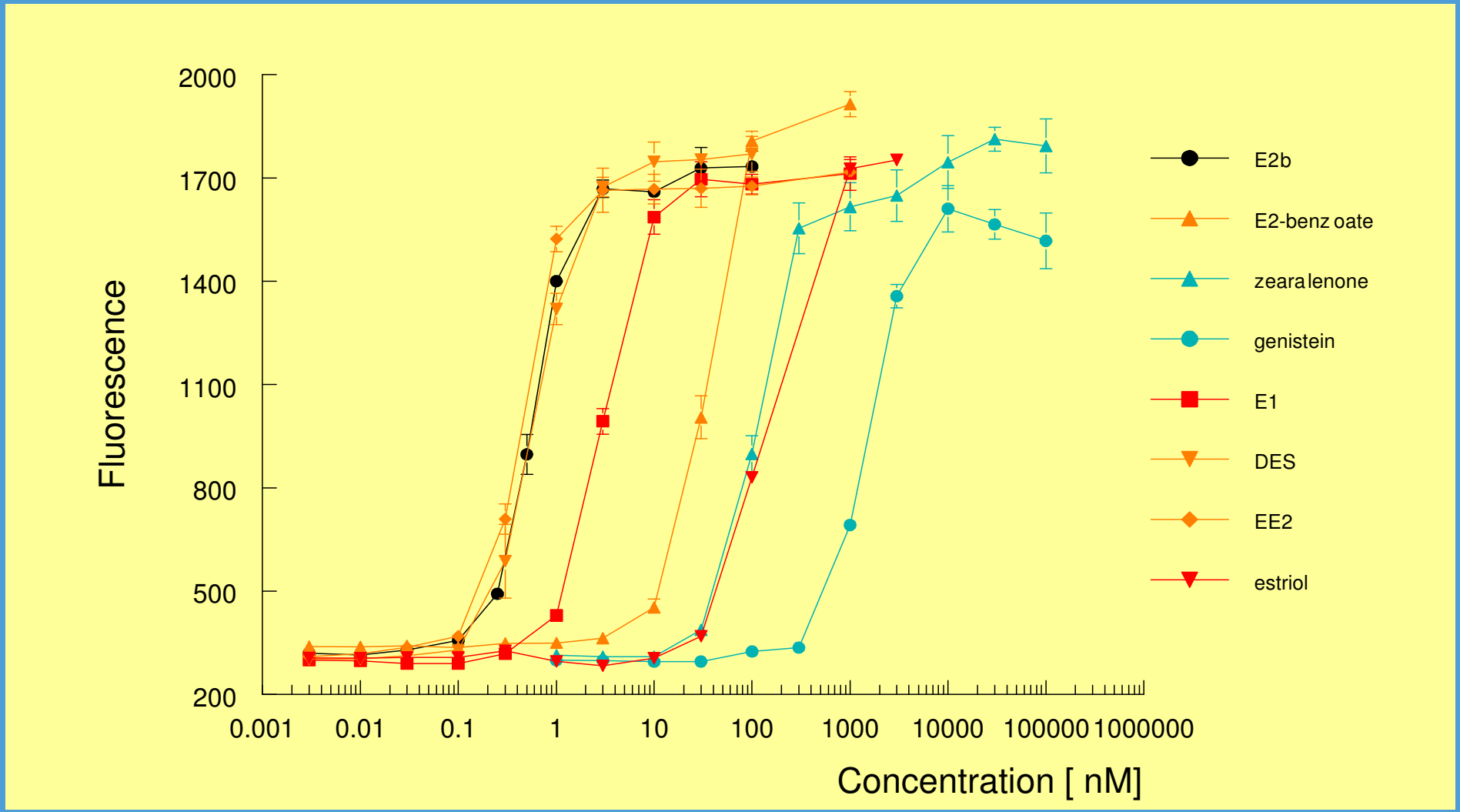
The yeast estrogen bioassay



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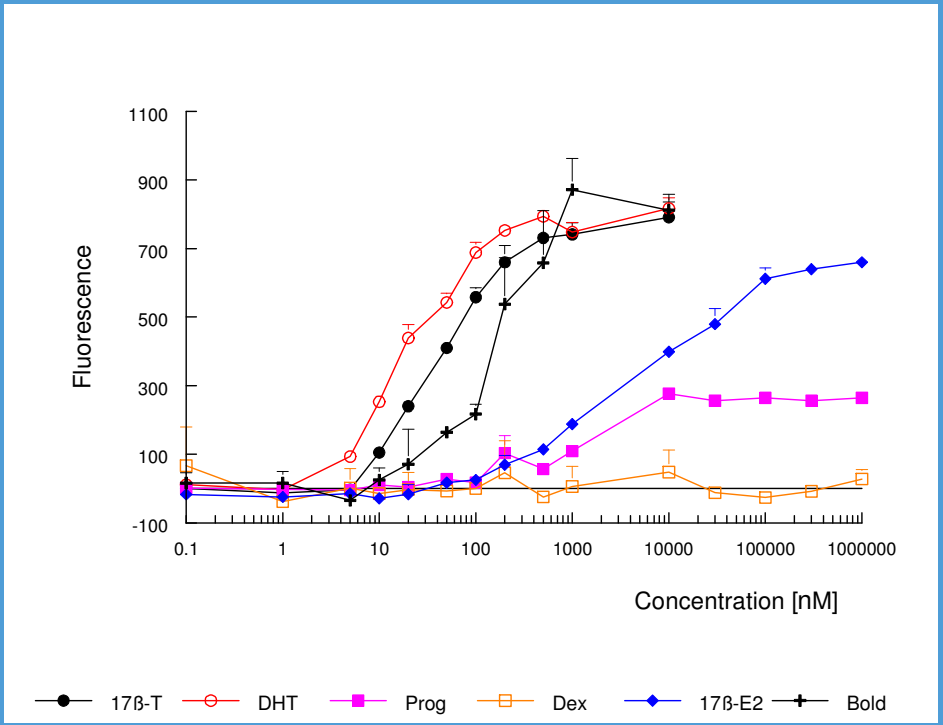
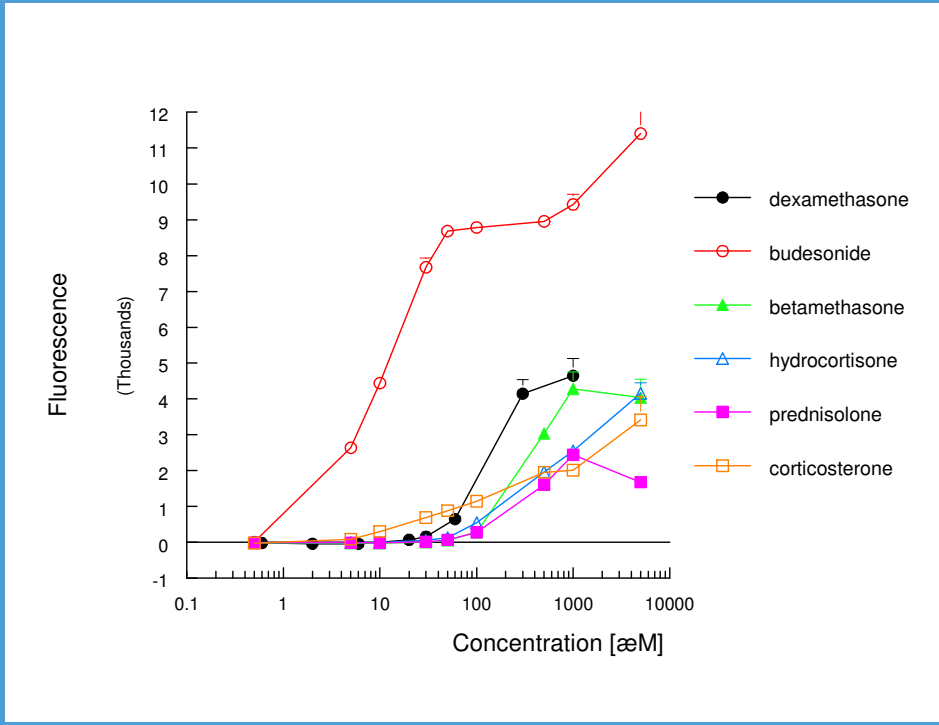
Bovee et al., *Gene* **325** (2004) 187-200
Bovee et al., *JSBMB* **91** (2004) 99-109

Similarly we developed a yeast androgen bioassay and yeast corticoid bioassay





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Bovee et al., *ABC* **389** (2007) 1549-1558
Bovee et al., *ABC* **401** (2011) 873-882

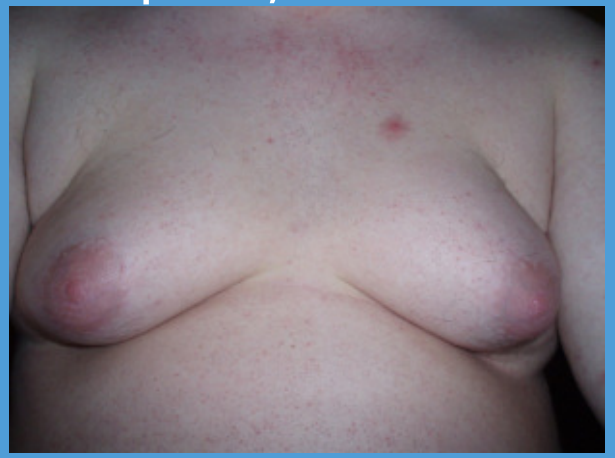
I am not going to show you that:

- **SERMs** and **SARMs** show their specific responses in these yeast hormone bioassays too
- Both the yeast estrogen and androgen bioassay were **fully validated** for both the screening of **feed** and **calf urine** samples (according to Directive 2002/657/EC and accredited ISO 17025)
- The yeast estrogen bioassay performed well in an **inter-laboratory ring test** with **calf urine** samples
- Was shown a cheap alternative for real practise: estrogen bioassay **screening calf urine samples vs GC-MS analysis**

Bovee et al., *JSBMB* **118** (2010) 85-92
Bovee et al., *ACA* **529** (2005) 57-64
Bovee et al., *FAC* **23** (2006) 556-568
Bovee et al., *ACA* **637** (2009) 225-234
Bovee et al., *ACA* **637** (2009) 265-272
Nielen et al., *FAC* **23** (2006) 1123-1131

I am also not going to show you that:

- **A 'natural' herbal supplement** for prostate problems, causing **gynaecomastia** in a 67 year old man, was screened with the yeast estrogen bioassay and that it turned out that the supplement contained **DES** (Geldrop Hospital, The Netherlands) (This morning Dr. Weigel)



- The yeast androgen bioassays specifically indicated the **anti-androgenic potential of the printing ink compound 2-isopropylthioxanthone (ITX),** which was confirmed *in vivo* (rat) (food packaging)

- Was **validated by Waternet/Waterproef** Laboratorium in The Netherlands for screening **estrogens in water** samples

Tooriaans et al., *FAC* **27** (2010) 917-925
Peijnenburg et al., *TiV* **24** (2010) 1619-1628
Nguyen et al., *TiV* **25** (2011) 2003-2009

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And I am also not going to show you that:

- The yeast estrogen and androgen bioassay are successfully used at **Ghent University for the screening of food supplements** (including bio-directed identification)

- Both the yeast estrogen and androgen bioassay are used as the department of **Food Chemistry, WUR** for **screening and bioassay-directed identification of active compounds in soy and licorice root**

- Both bioassays validated and used for calf urine and feed at the **National Veterinary Research Institute, Poland**

- Both bioassays used for the screening of calf urine samples at the University of Veterinary Medicine, Turin, Italy

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Becue et al., *ABC* **339** (2011) 1031-1039
Simons et al., *ABC* **401** (2011) 305-313
Simons et al., *J. Agric. Food Chem.* **59** (2011) 6748-6758
Divari et al., *FAC* **27** (2010) 1123-1131

Nor am I going to show you that:

- The yeast estrogen is successfully used at GSF, National Research Centre for Environment and Health, Neuherberg, Germany (**Sediments**)
- The yeast (gluco)corticoid assay was successfully used at Utrecht University-IRAS (**hydroxylated PCBs**)
- The yeast estrogen bioassay was successfully used at Utrecht University-IRAS (**seawater contaminated with oil**)
- Is used at 1) Oregon State University, USA; 2) Amherst College, Chemistry Department, USA; 3) McGill University, Montreal Canada; 4) Chinese Academy of Science, China; 5) KFRI, Korea; 6) CRI, Bangkok, Thailand; 7) TU Dresden, Germany; 8) VITO, Belgium

Levy et al., *Environ. Sci. Pollut. Res. Int.* **18** (2011) 99-110
Antunes Fernandes et al., *Toxicology Letters* **206** (2011) 185-165
Vrabie et al., *Environ. Toxicol. Chem.* **29** (2010) 1529-1536

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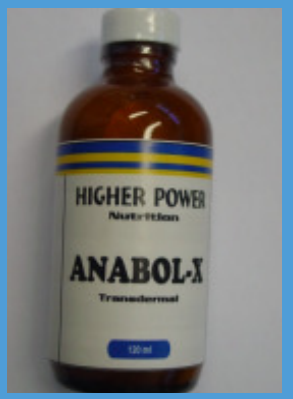
Nor that....

- RIKILT: The yeast androgen assay was shown to of **added value** in a study with dietary supplements: a comparison with a liquid chromatography tandem mass spectrometry (LC-MS/MS) method (**this morning Dr. Weigel**)

- RIKILT: Showed an **added value** by the identification of anabolic steroids and derivatives in supplements, using bioassay-guided fractionation, UHPLC/TOMMS analysis and accurate mass database searching



Rijk et al., *ACA* **637** (2009) 305-314
Peters et al., *ACA* **664** (2010) 77-88

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But I am going to show you:

- Approach of effect screening will work for marine toxins as well

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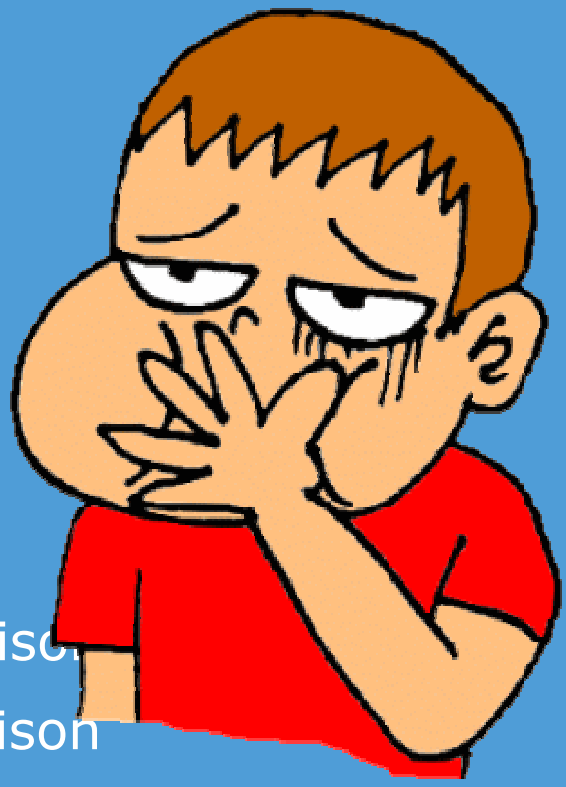
Introduction

- Food poisoning
  - Bacteria
  - Viruses
  - Toxins



  - Marine toxins
    - ASP – Amnesic Shellfish Poison
    - PSP – Paralytic Shellfish Poison
    - NSP – Neurotoxic Shellfish Poison
    - DSP – Diarrheic Shellfish Poison

Introduction

- Rats and mice are used for detecting marine toxins in mussels
  - By feeding
  - By injection

- From 2015 these methods will be forbidden (however not for testing production areas)
- LC-MS/MS method is available (for lipophilic toxins)
  - Expensive
  - Not high-throughput
  - Not developed to detect all marine toxins and unable to detect unknown toxins

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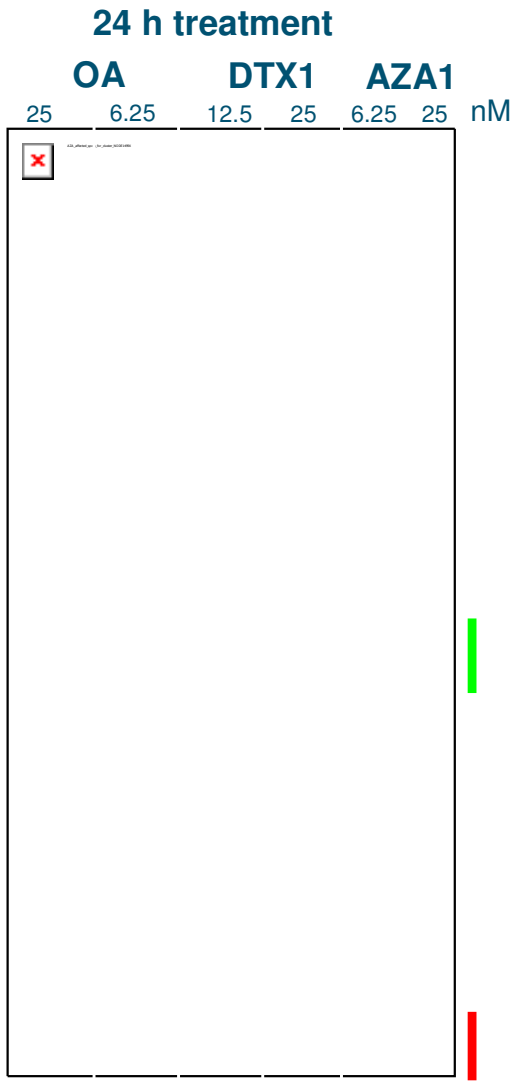
I Development of a dedicated array for the detection of marine toxins. From genomics, via a dedicated tube array, back to the development of a specific bioassay?

- Gene Selection (RIKILT): Caco-2 exposed to dinophysis toxin-1 (DTX1), azaspiracid 1 (AZA1) and okadaic acid (OA)
    - Whole genome microarray (Service XS)
        - Data analysed for selection marker genes

Gene selection (Caco-2: OA, DTX1 and AZA1)



Red: upregulated
Green: downregulated

AZA specifically down

AZA specifically up

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Marine toxins – Genomics - Exposure of human Caco-2 cells

- Genes specifically up- or down-regulated DTX1, AZA1 or OA were selected and used to develop a dedicated array (BioCop).

Gene	EXPRESSION GROUP	Spiked AZA1 AVR	Spiked DTX1 AVR	INT_AVR vs. median	Intensity rank on 1 to 100
C21orf129	AZA and DTX1 down; BI_Mus_0	-1.76	-1.545	17.8	79
TFEC	AZA and DTX1 down; BI_Mus_0	-1.55	-0.95	2.5	43
C3orf57	Uniquely down by AZA1	-1.75	-0.12	4.7	56
NPPB	Spec down by DTX1	-0.07	-1.82	52.3	90
MT1H	Uniquely up by AZA1, but also by higher DTX1 doses	2.155	0.23	22.3	82
MT1G	Uniquely up by AZA1, but also by higher DTX1 doses	2.375	0.23	16.4	78
CDKN1C	Uniquely up by DTX1	-0.42	2.7	8.4	67
VASN	Uniquely up by DTX1	-0.06	3.1	4.6	55
MAFB	Uniquely up by DTX1	0.09	3.495	1.7	34
RGS16	Uniquely up by DTX1	0.3	2.79	1.7	34
LOC387763	DTX1_>4x_AZA1_1,4_to_1.7_up	0.6	3.515	1.0	22
TUBB3	DTX1_>4x_AZA1_1,4_to_1.7_up	0.715	2.245	23.4	83
CEACAM1	AZA1 AND DTX1 UP	2.135	1.505	22.0	82
TNS4	AZA1 AND DTX1 UP	2.66	1.795	1.3	28
DDIT4	AZA1 AND DTX1 UP	2.4	1.435	18.7	80
TMCC1	Exp_Maria_AZA1_2,8up_DTX_max_1,3up	1.155	0.14	1.9	37
MT1F	Exp_Maria_AZA1_2,8up_DTX_max_1,3up	1.355	-0.5	11.0	72
TRIB3	Exp_Maria_AZA1_2,8up_DTX_max_1,3up	1.11	0.06	4.7	56
OSR2	Exp_Maria_AZA1_2,5down_DTX_max_1,4down	-0.845	0.81	2.8	46
AK091132	Exp_Maria_AZA1_2,5down_DTX_max_1,4down	-1.505	-0.175	3.4	50
GAPDH	**Control**	0.13	-0.14	318.9	99
TMEM179B	**Control**	-0.04	0.04	3.5	50

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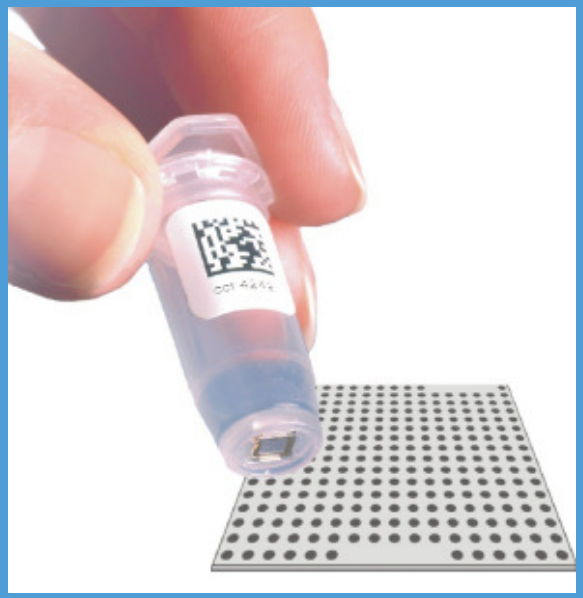
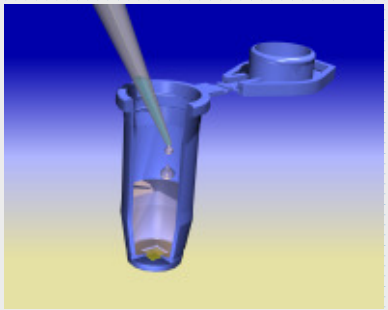
The newly developed Array Tube (Alere)

Transcriptomics assay on Clondiag AT-Platform >> ArrayTube (AT) Platform



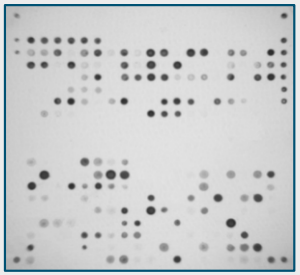
single tube format
based on conventional
laboratory vials
(Clondiag is nowadays
Alere)

microtube

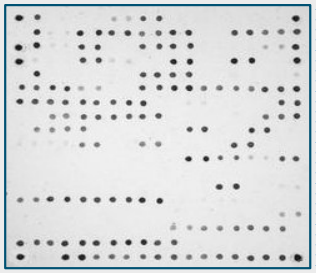


- easy processing with standard lab equipment
- no evaporation
- uniform wettability
- small volumes
- optimal processing through small surface area

+ 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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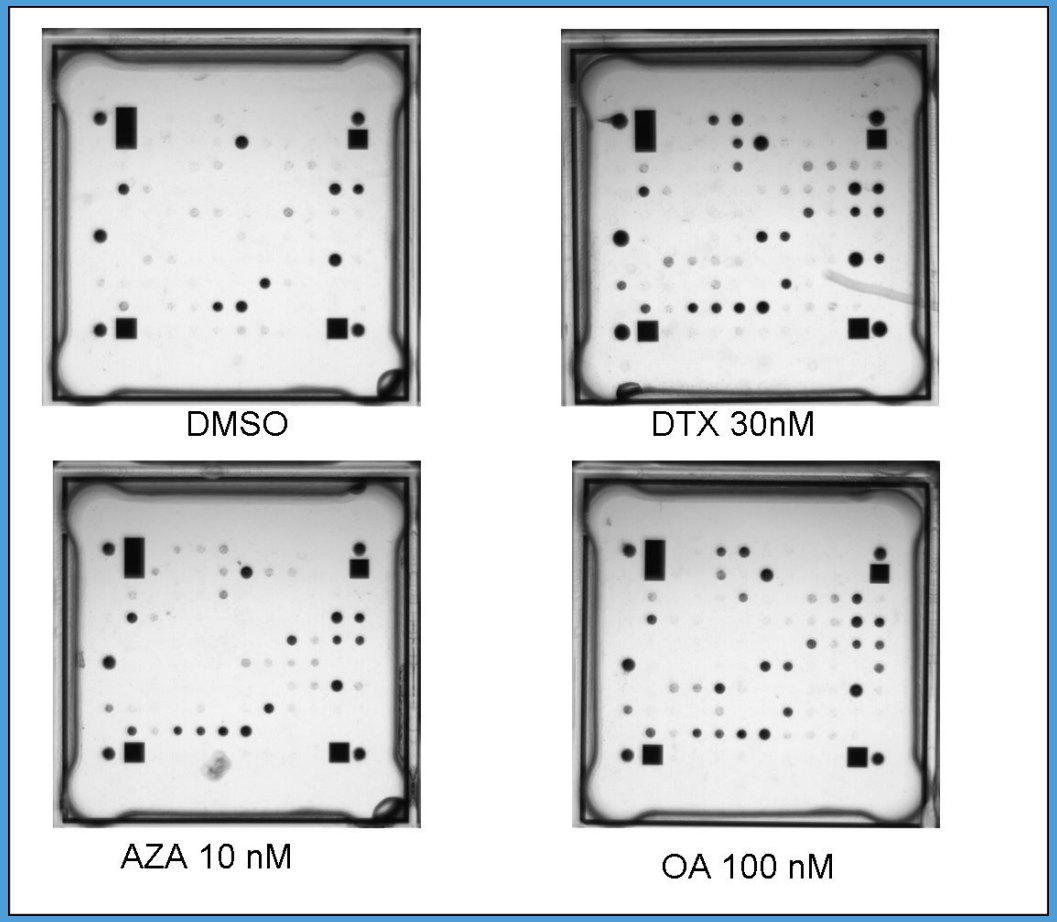
Array Tube Marine Toxins

- Expose Caco-2 cells to standards or extracts (2-24h) and isolate the mRNA
- **Synthesize** the corresponding **cDNA** with a primerset of the 20 selected marker genes and 2 control genes (GAPDH and TMEM) (**primerset 1**)
- **Linear amplification with biotin labelled primerset 2** (in presence of competitor primer set 3, complementary to primers in set 1 and with an aminolink, in order to prevent an exponential amplification)
- **Hybridization on the Array Tube** (staining was performed with a streptavidin coupled with a peroxidase and the addition of tetramethylbenzidin. The peroxidise activity catalyses the conversion of TMB and the dark blue precipitate product is measured by an array tube reader)
- **Measuring and data management (software) to get the results**

Marine toxins – Development of a dedicated low-density microarray for genomic profiling



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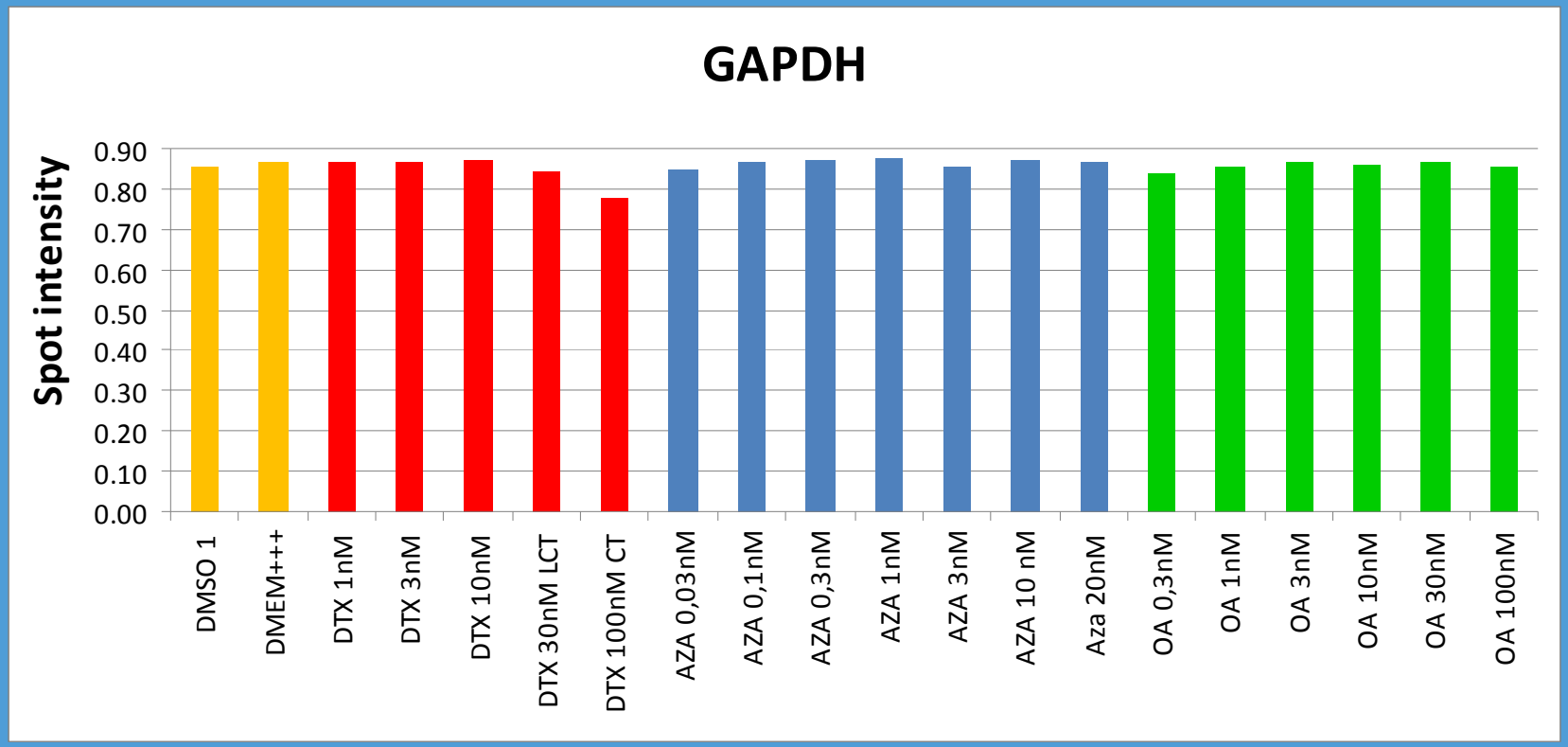
Bovee et al., *ES&T* **45** (2011) 8965-8973

Expression profile of the GAPDH control gene in the newly developed Array Tube



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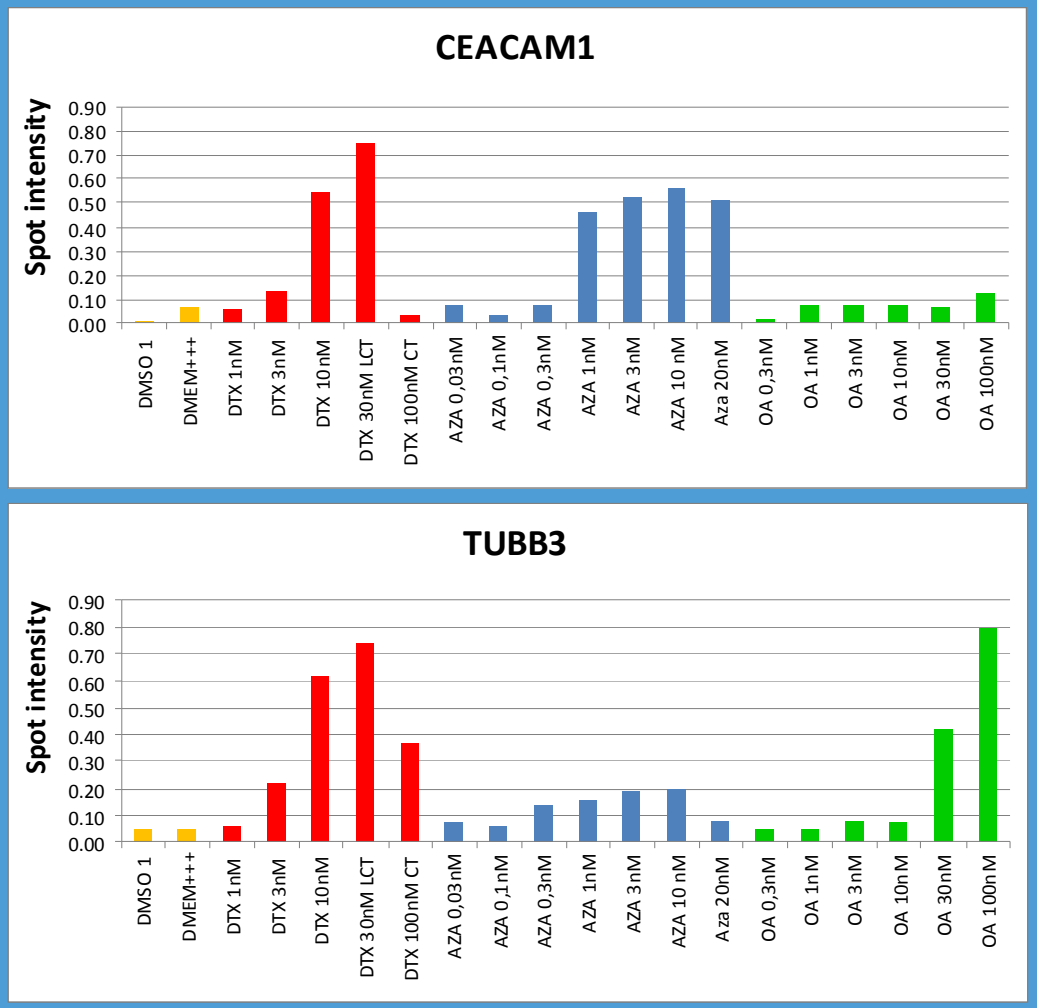
Expression profile of the CEACAM1 and TUBB3 marker genes in the Array Tube



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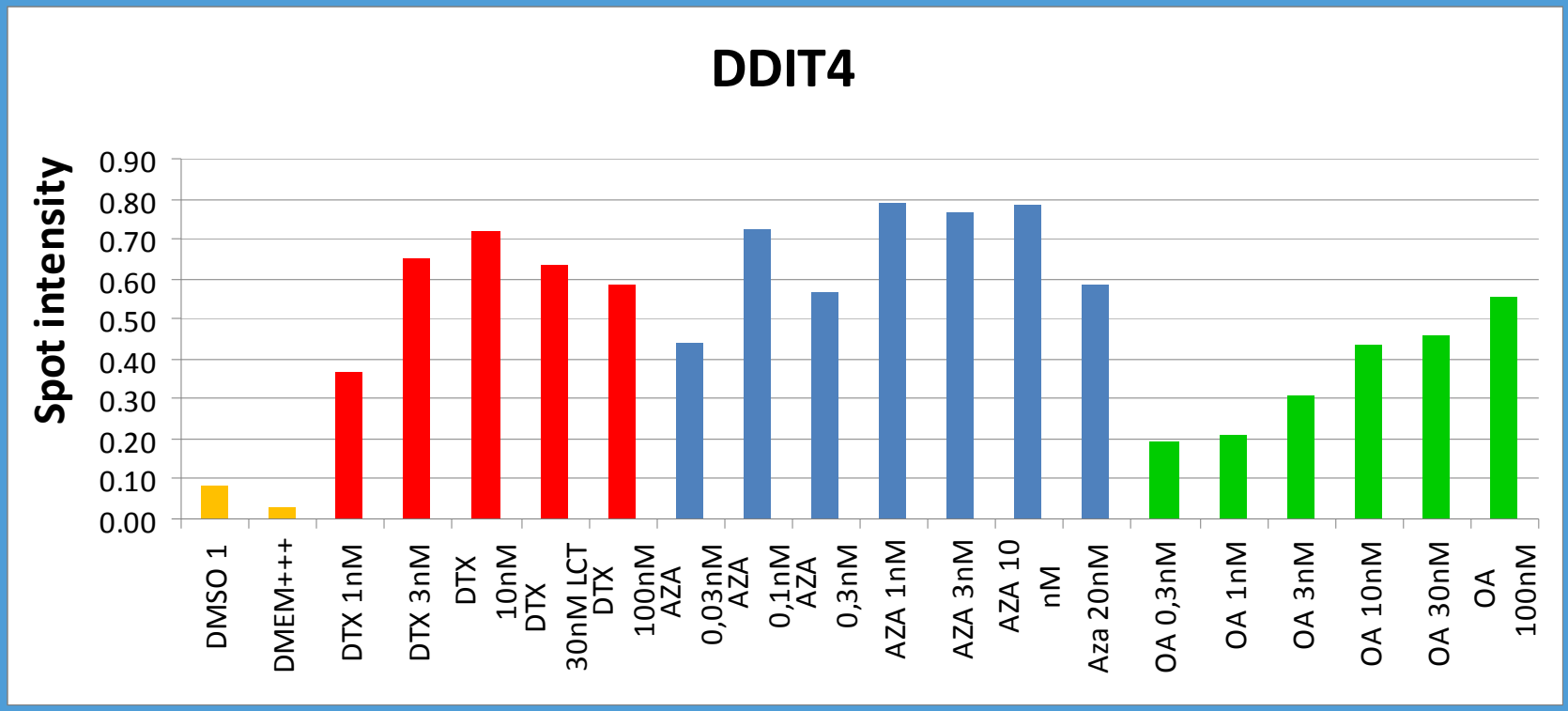
Expression profile of the DDIT4 marker gene in the Array Tube



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Bovee et al., *ES&T* **45** (2011) 8965-8973

Newly developed Array Tube for the detection of marine toxins

- Thanks to the BioCop EU project (Prof. Elliott, QUB), Alere and the help from the group of Prof. Dr. Naegeli (Zürich)
- It works, but....not all of the selected genes gave responses (7/20)
- Labour intensive
- Not cheap
- Still takes about 3 days (mouse assay 1 day)

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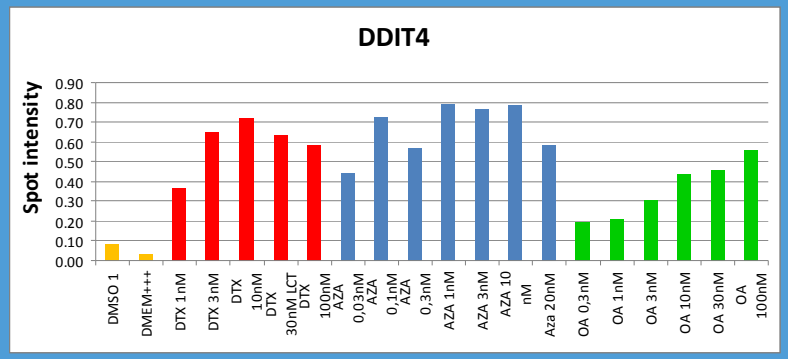
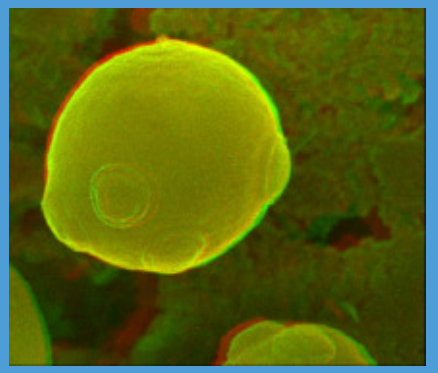
Alternatives – Bioassays or Molecular beacons or Multiplex qRT-PCR





- 1 Synthesize the -2.5 kb 5’ from the ATG start of the DDIT gene (P) and combine with a marker (e.g. Luc or GFP) in a suited vector
- 2 E.g. Transfect the Caco-2 cell line with the DDITpromoter-Luc vector
- 3 Select clones on luciferase expression upon exposure to 1 nM AZA1

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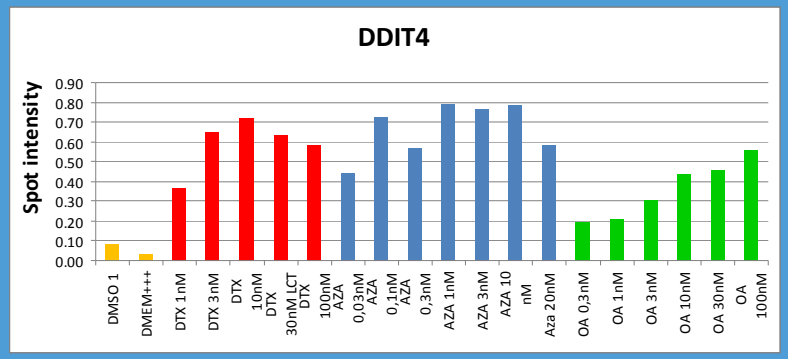
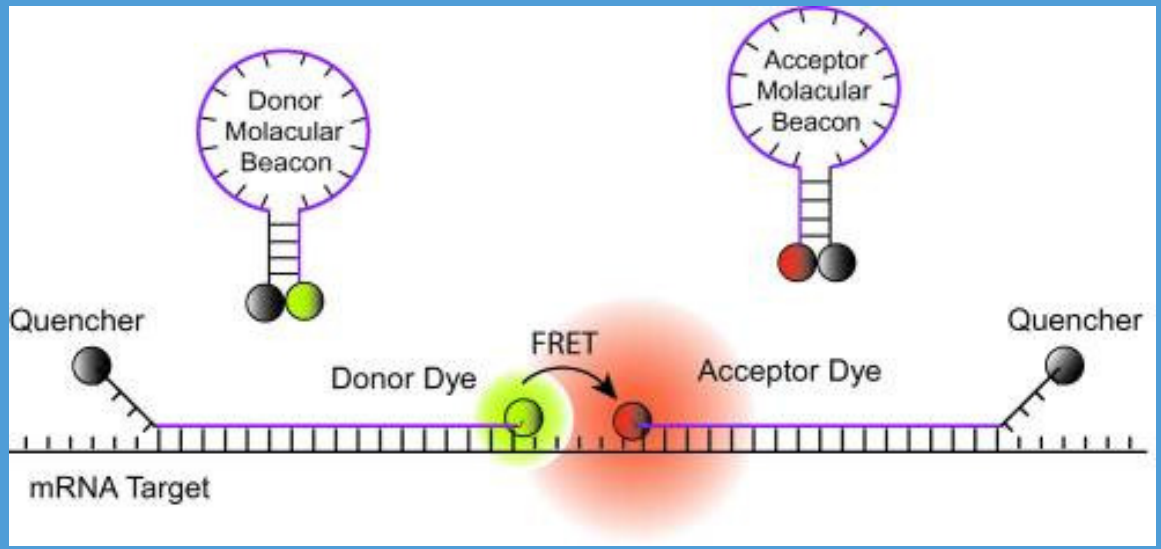
Alternatives – Bioassays or Molecular beacons

or Multiplex qRT-PCR



A schematic illustration showing the concept of dual FRET molecular beacons. Hybridisation of donor and acceptor molecular beacons to adjacent regions on the same mRNA target results in FRET between donor and acceptor fluorophores upon donor excitation. By detecting FRET signal, fluorescence signals due to probe/target binding can be readily distinguished from that due to molecular beacon degradation and non-specific interactions [Santangelo et al., *Nucleic Acids Research* **32** (2004) 1-9].



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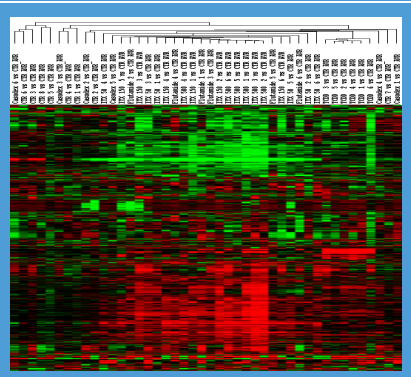
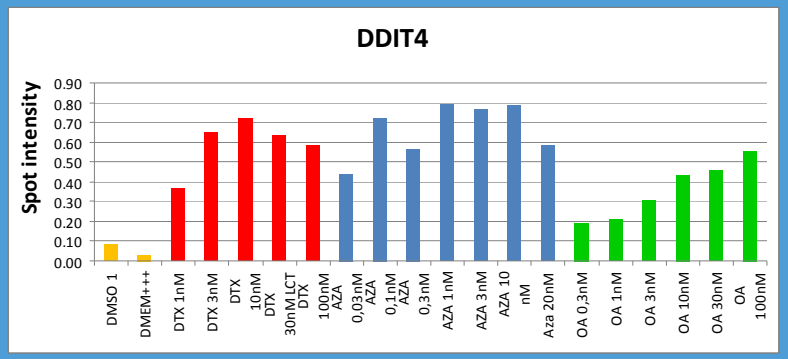
Alternatives – Bioassays or Molecular beacons or Multiplex qRT-PCR





⇒ ⇐ qPCR

- 1 Take the Caco-2 cell
- 2 Expose cells and isolate mRNA
- 3 Perform qPCR on selected marker genes, e.g. DDIT4, CEACAM1, TUBB3, TRIB3 and OSR2

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Gene Selection for qRT-PCR

- Based on the first exposure
- 22 genes were selected for the development of the Array Tube (DTX1, AZA1, OA)
- The complementary commercially designed Primers were ordered at Qiagen
- All tested individually in a SYBR green qRT-PCR (all worked well)

- 2nd exposure Caco-2 to yessotoxin (YTX) and pectenotoxin (PTX)
    - Whole genome microarray
        - Data analysed for selection of marker
        - All tested individually in a SYBR green qRT-PCR

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Qiagen – Primers and Probe design

- TMEM179B -> TTTACTCCAACCTACAC
- RGS16 -> CCGCCTTCCCCACCAC
- DDIT4 -> TGTGTTTGTTGTTTGTT
- NPPB -> CACCACGAAGCCCCAA

TMEM179B used as reference gene

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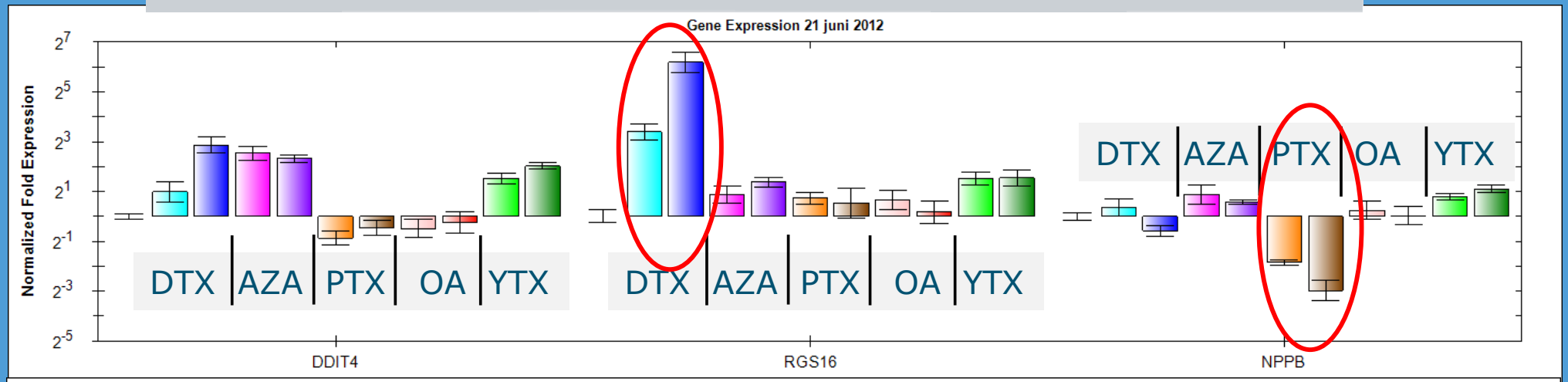
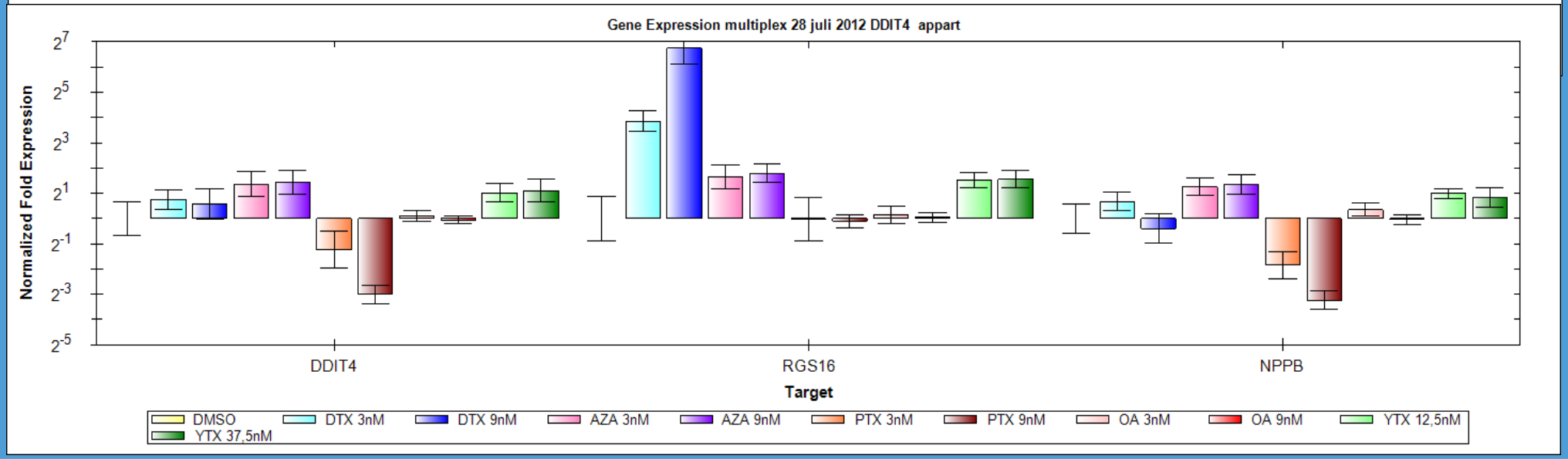
RIKILT – single PCRs used to attribute the Probes

gene	dye	Excitation(nm)	Emission nm)
TMEM179B	-> Cy5	643	667
RGS16	-> Texas Red	596	615
DDIT4	-> Hex	535	553
NPPB	-> 6Fam	494	518

Results- “Singleplex” qRT-PCR (Biorad Sybr Green) & Multiplex qRT-PCR (Qiagen Quantifast Multiplex)

	DTX	AZA	PTX	OA	YTX
DDIT4	↑↑↑	↑↑↑	↓↓↓	? ↓↓	↑↑↑
RGS16	↑↑↑	-↑↑	? ↑↓	? ↑↓	? ↑↑
NPPB	↓↓↓	-↑-	↓↓↓	?-↓	? ↑↑





Present

- Able to detect ADTX, AZA, PTX and YTX
- Able to distinguish DTX and PTX

Future work

- Developing the second multiplex qRT-PCR with the selected marker genes: CXCR4, EGR1 and TGFB2
  - Detect OA
  - Distinguish between AZA and YTX

	DTX	AZA	PTX	OA	YTX
CXCR4	↑	?	?	↑	?
EGR1	?	?	?	↑	?
TGFB2	↓	↓	?	↓	?

TMEM179B used as reference gene

Future work

- Testing with blank and incurred mussel extracts (including positive in mouse bioassay, but negative by MS)
- Optimizing the method
    - Shorter exposure time
    - Faster RNA isolation kit
- Validation study
- Expand to other marine toxins (e.g. STX and SPX)
- Compare outcomes with a method being developed by a PhD student: using murine embryotic stem cell derived beating cardiomyocytes