

Stiftung Tierärztliche Hochschule Hannover
University of Veterinary Medicine Hannover, Foundation



Assessment of mixture effects of estrogenic and anti-androgenic pesticide residues at low, consumer-relevant concentrations *in vitro*

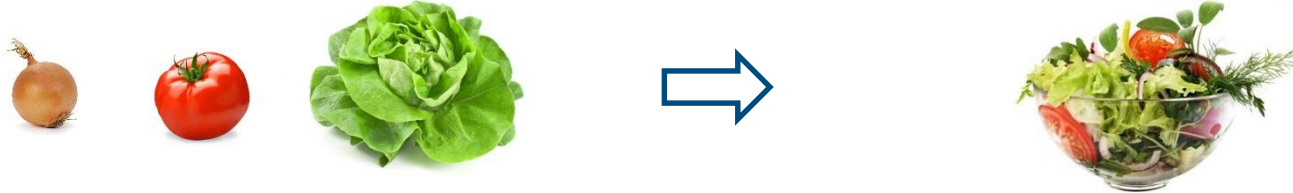
10th BioDetectors Conference in Sorrento, Italy
7th April 2017



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Institute for Food Toxicology, University of Veterinary Medicine Hannover, Foundation

Exposure to pesticide mixtures and their current risk assessment



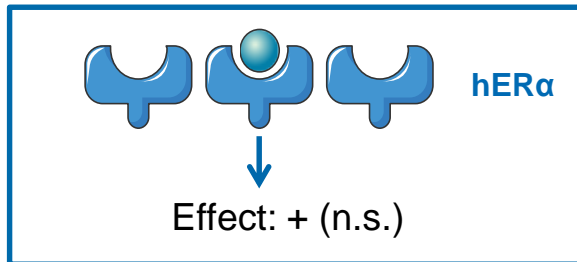
1/3 multiple pesticide residues (2-10)

low concentrations of a multitude of residues

EFSA., EFSA Journal (2015)

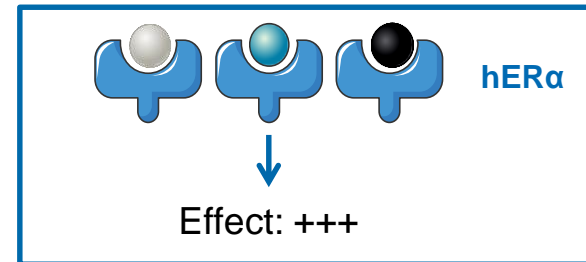
Pesticide residues are consumed in a mixture, individually occurring at low concentrations.

Single pesticide residue
low concentration



current risk assessment
maximum residue levels (MRL) for single substances

multiple pesticide residues
low concentration
same target



new approach:
cumulative assessment groups



Aim and scope of the study

Evaluation of the effects of **pesticide mixtures**
activating the hER α and hER β or **inhibiting the hAR**
at **low, food consumer-relevant concentrations *in vitro***

screening in different *in vitro* test systems
yeast-based and human cell-based

Use of the Concentration Addition (CA) model



cumulative risk assessment for estrogenic/anti-androgenic pesticides

Selection of test substances and mixture components

Estrogenic pesticides

• fludioxonil		×
• fenhexamid		×
• chlorpyrifos		×
• fenarimol	×	×
• pirimicarb		×
• propamocarb		×
• 2,4'-DDT	×	×
• 4,4'-DDT	×	×

Anti-androgenic pesticides

• procymidone	×	×	×
• vinclozolin	×	×	×
• tebuconazole			×
• propiconazole			×
• fenarimol	×	×	
• prochloraz			×



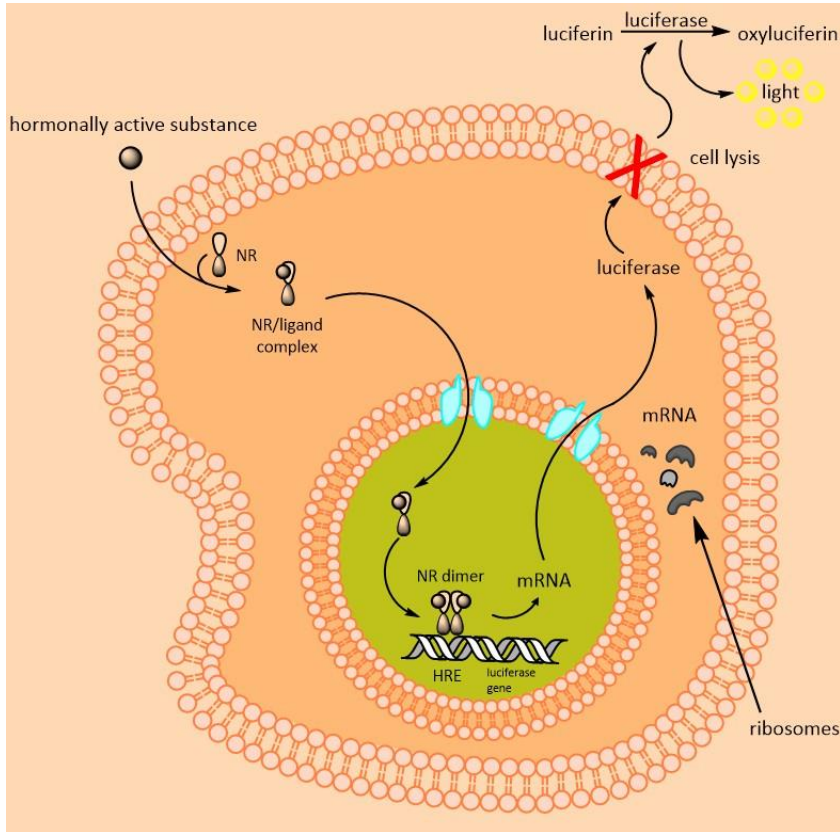
- × not approved in the EU
- × not approved but found as residues
- × approved and frequently used
- × well-described reference substances

food **consumer-relevant** pesticide mixtures were tested with well-described reference substances.

Test systems

AR/ER α / β CALUX

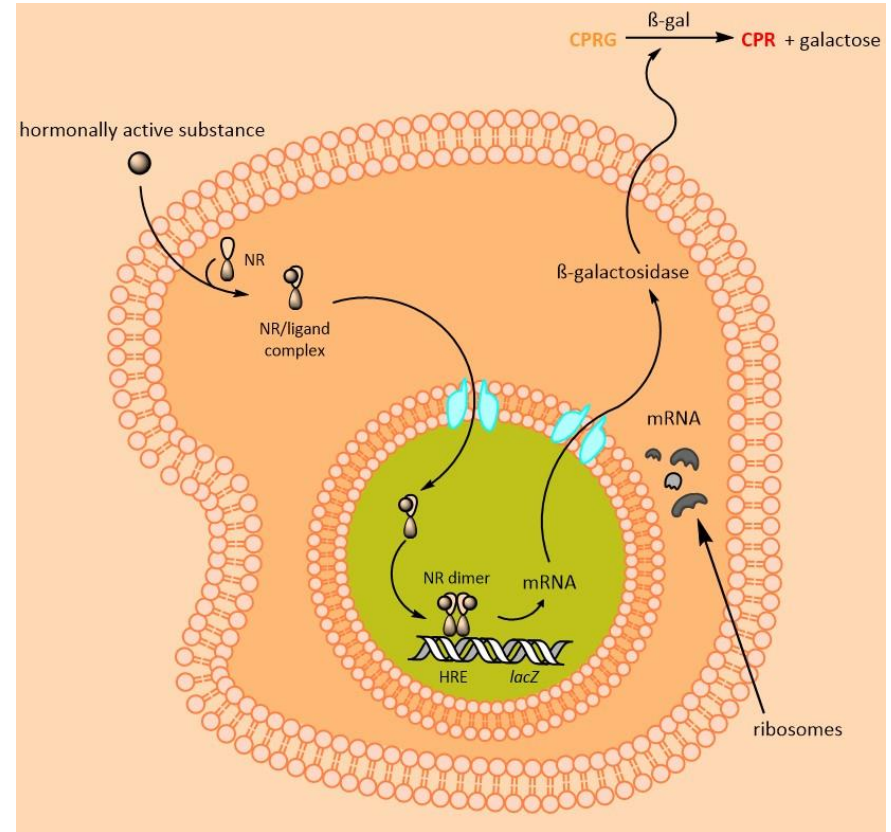
Chemically Activated LUCiferase gene eXpression



Used according to protocols of BDS
(n=3 with triplicates in 96 well plates)

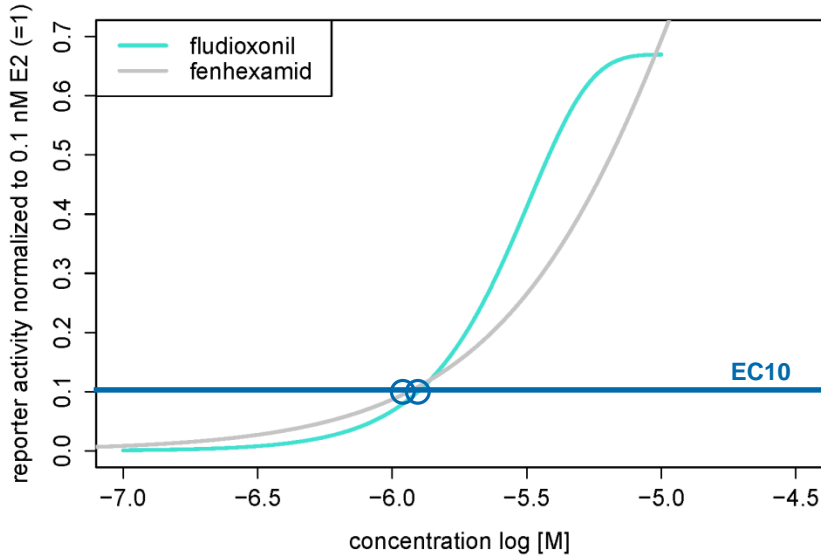
YAS/YES

Yeast-based Androgen/Estrogen Screen



Used according to protocols of BASF
(n \geq 5 with quadruplicates in 96 well plates)

Mixtures



prediction: Concentration Addition (CA)

Loewe and Muischnek., Archiv f. Experiment. Pathol. u. Pharmacol. (1926)

Analysis software developed by Prof. Frank Klawonn

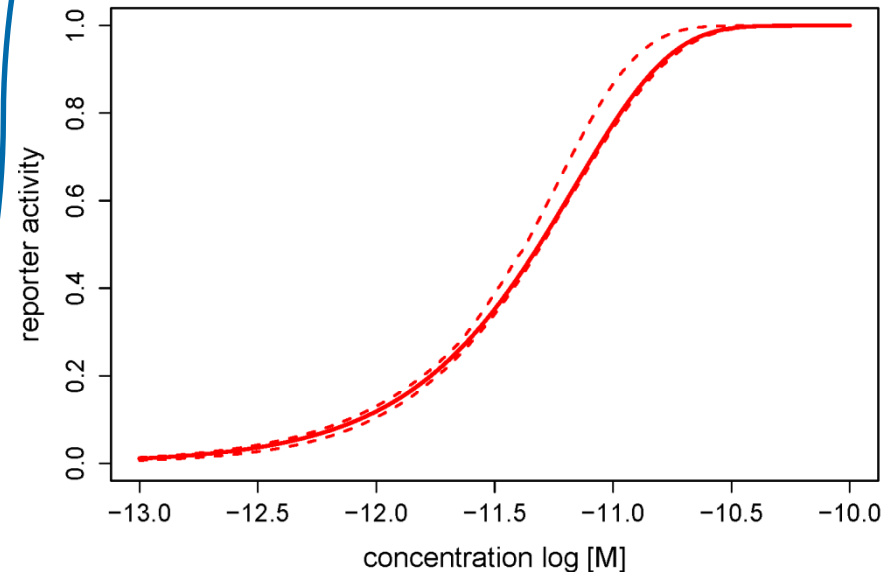
experiment

Comparison of predicted and observed data

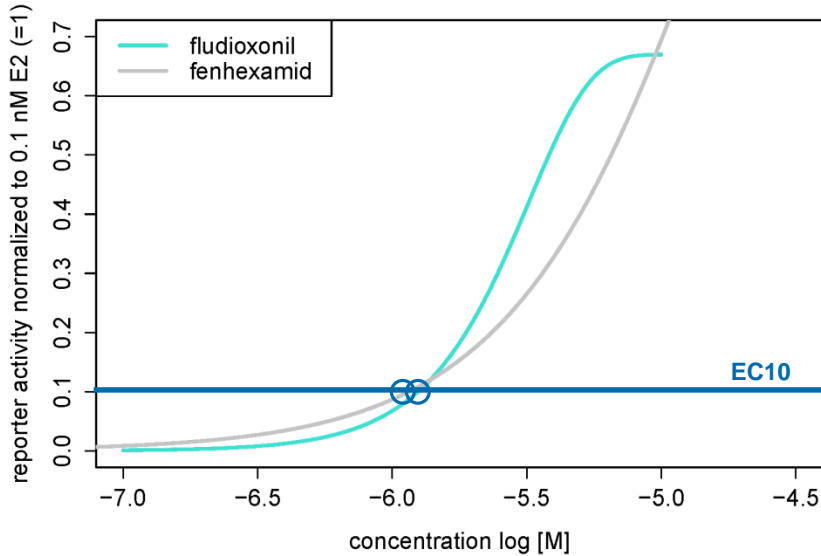
binary and ternary
iso-effective mixtures of
estrogenic or anti-
androgenic pesticides

substance	EC10	mixture ratio
fludioxonil	1.3 μ M	32%
fenhexamid	2.6 μ M	68%
	3.9 μ M	100%

dilution series



Mixtures



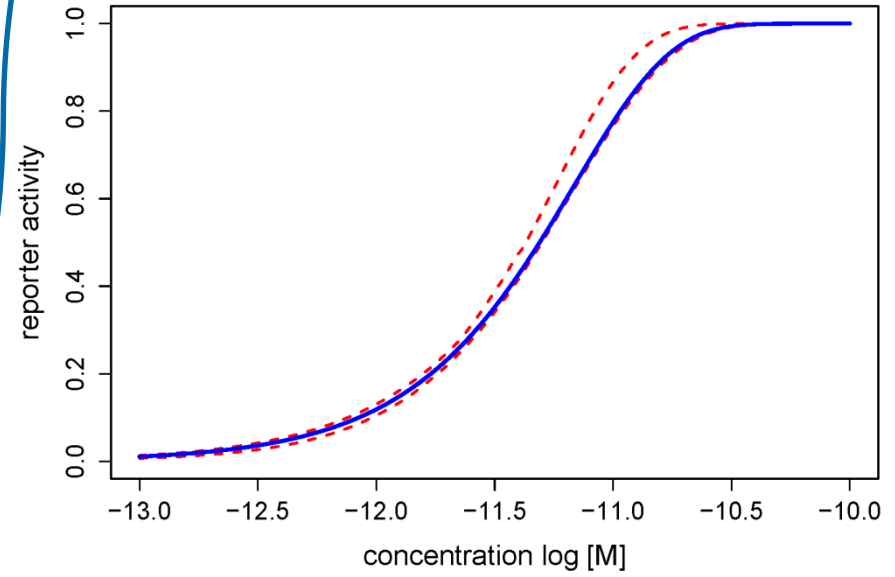
prediction: Concentration Addition (CA)

Loewe and Muischnek., Archiv f. Experiment. Pathol. u. Pharmakol. (1926)
 Analysis software developed by Prof. Frank Klawonn

experiment

Comparison of predicted and observed data

Additivity

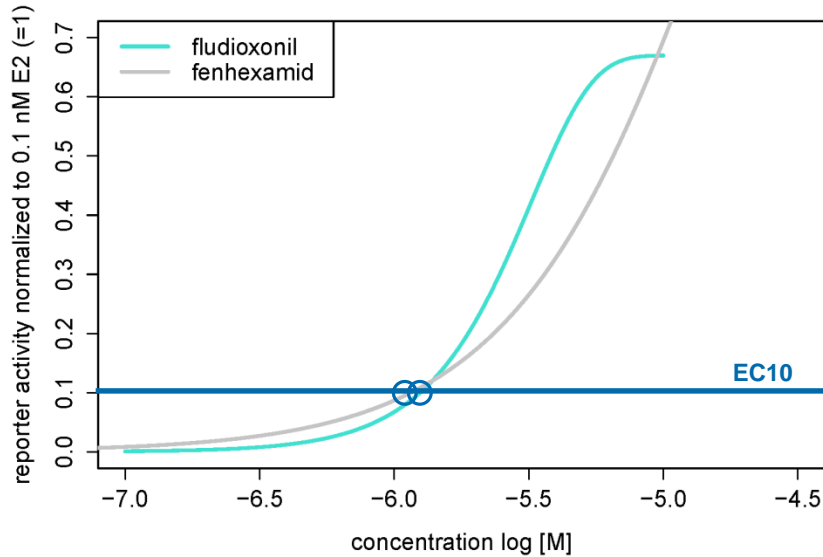


binary and ternary
iso-effective mixtures of
 estrogenic or anti-
 androgenic pesticides

substance	EC10	mixture ratio
fludioxonil	1.3 μ M	32%
fenhexamid	2.6 μ M	68%
	3.9 μ M	100%

dilution series

Mixtures



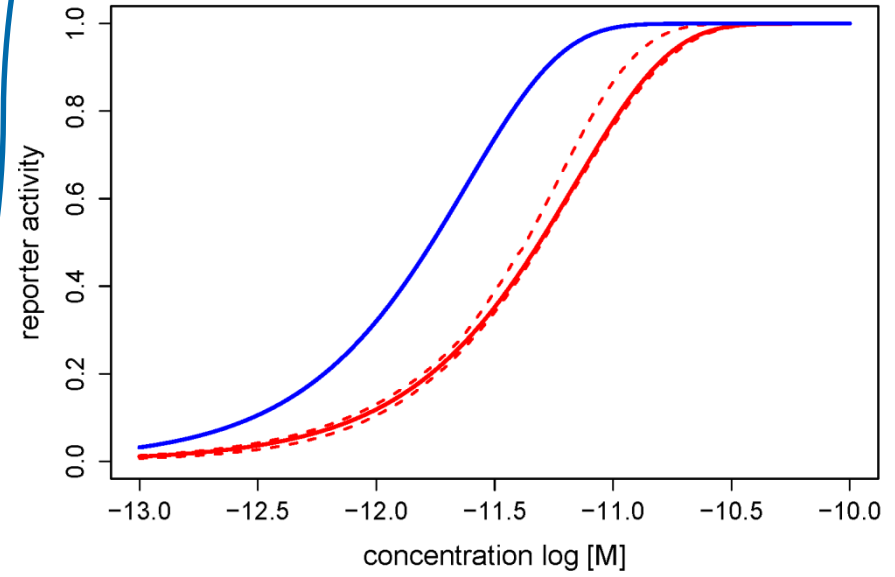
prediction: Concentration Addition (CA)

Loewe and Muischnek., Archiv f. Experiment. Pathol. u. Pharmacol. (1926)
 Analysis software developed by Prof. Frank Klawonn

experiment

Comparison of predicted and observed data

Sub-additivity

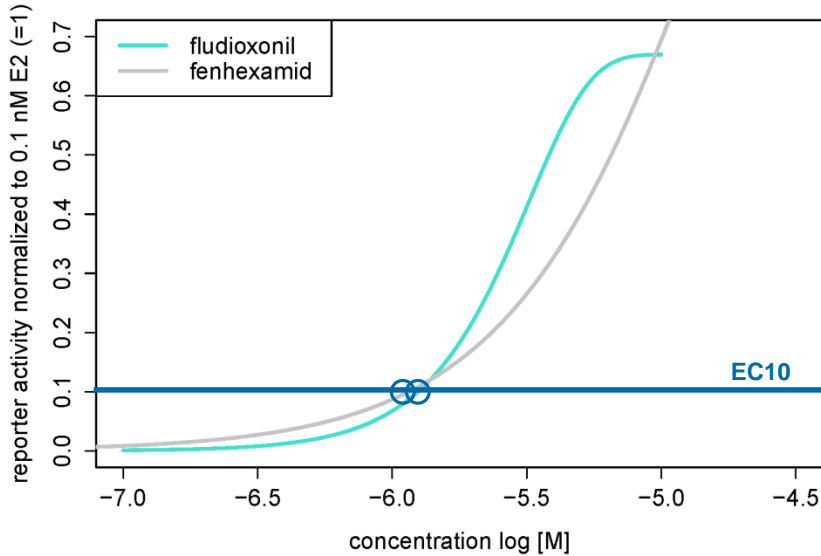


binary and ternary
iso-effective mixtures of
 estrogenic or anti-
 androgenic pesticides

substance	EC10	mixture ratio
fludioxonil	1.3 μ M	32%
fenhexamid	2.6 μ M	68%
	3.9 μ M	100%

dilution series

Mixtures



prediction: Concentration Addition (CA)

Loewe and Muischnek., Archiv f. Experiment. Pathol. u. Pharmacol. (1926)

Analysis software developed by Prof. Frank Klawonn

experiment

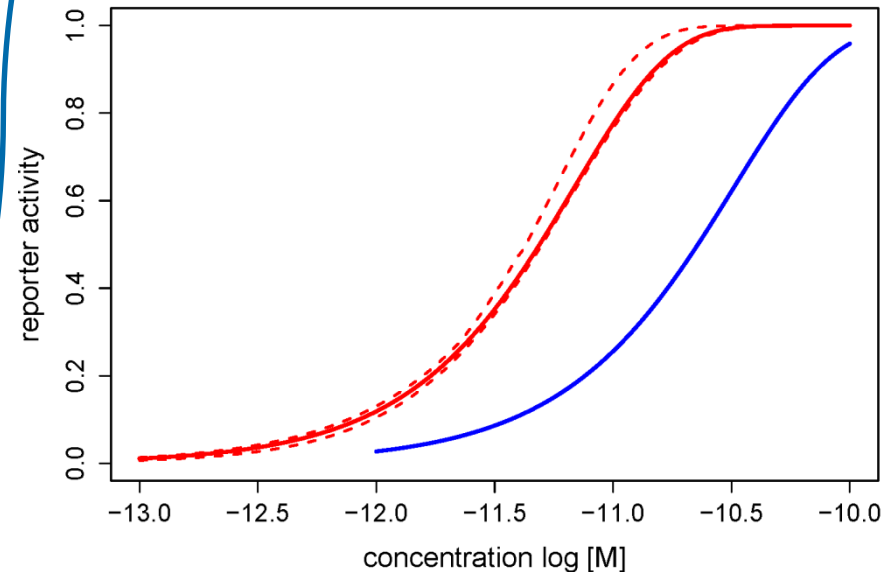
Comparison of predicted and observed data

binary and ternary **iso-effective** mixtures of estrogenic or anti-androgenic pesticides

substance	EC10	mixture ratio
fludioxonil	1.3 μ M	32%
fenhexamid	2.6 μ M	68%
	3.9 μ M	100%

dilution series

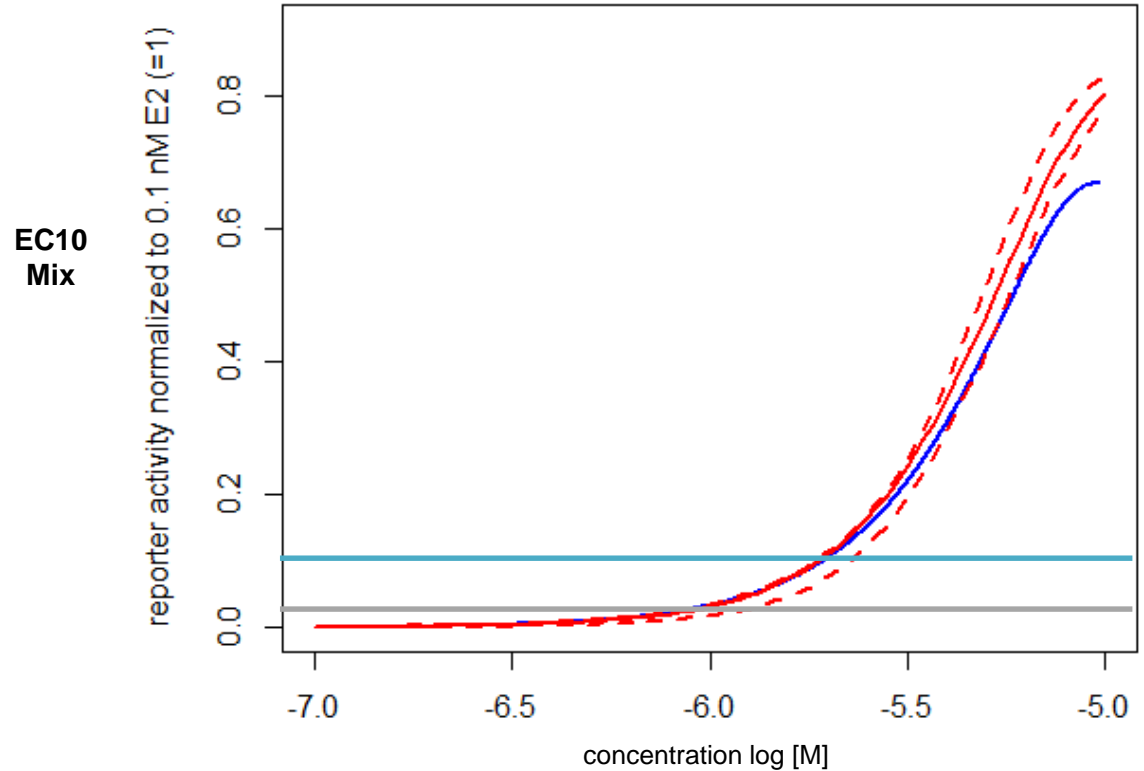
Synergism



Estrogenic mixtures - hER α

fludioxonil & fenhexamid

ER α CALUX



	predicted	observed [95% CI]
EC10	1.95 μ M	1.86 μ M [1.86 μ M-2.24 μ M]
EC01	0.50 μ M	0.55 μ M [0.49 μ M-0.78 μ M]

additive effects of fludioxonil and fenhexamid in YES and ER α CALUX

Estrogenic mixtures - hERα

1/1
1/10
10/1
10/10

EC01 mix – EC01
EC01 mix – EC10
EC10 mix – EC01
EC10 mix – EC10

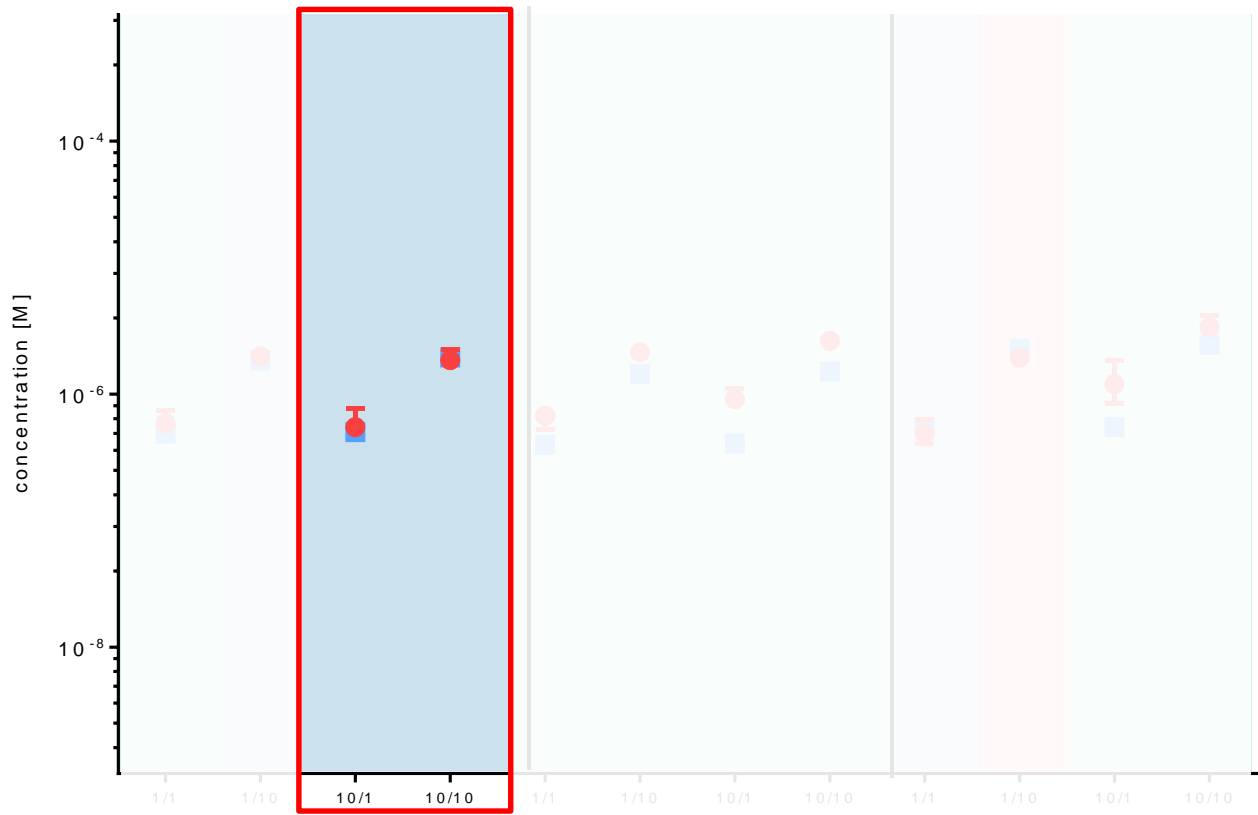
fludioxonil
fenhexamid

ERα CALUX

propamocarb
fludioxonil
fenhexamid

chlorpyrifos
fludioxonil
fenhexamid

additive



Estrogenic mixtures - hER α

1/1
1/10
10/1
10/10

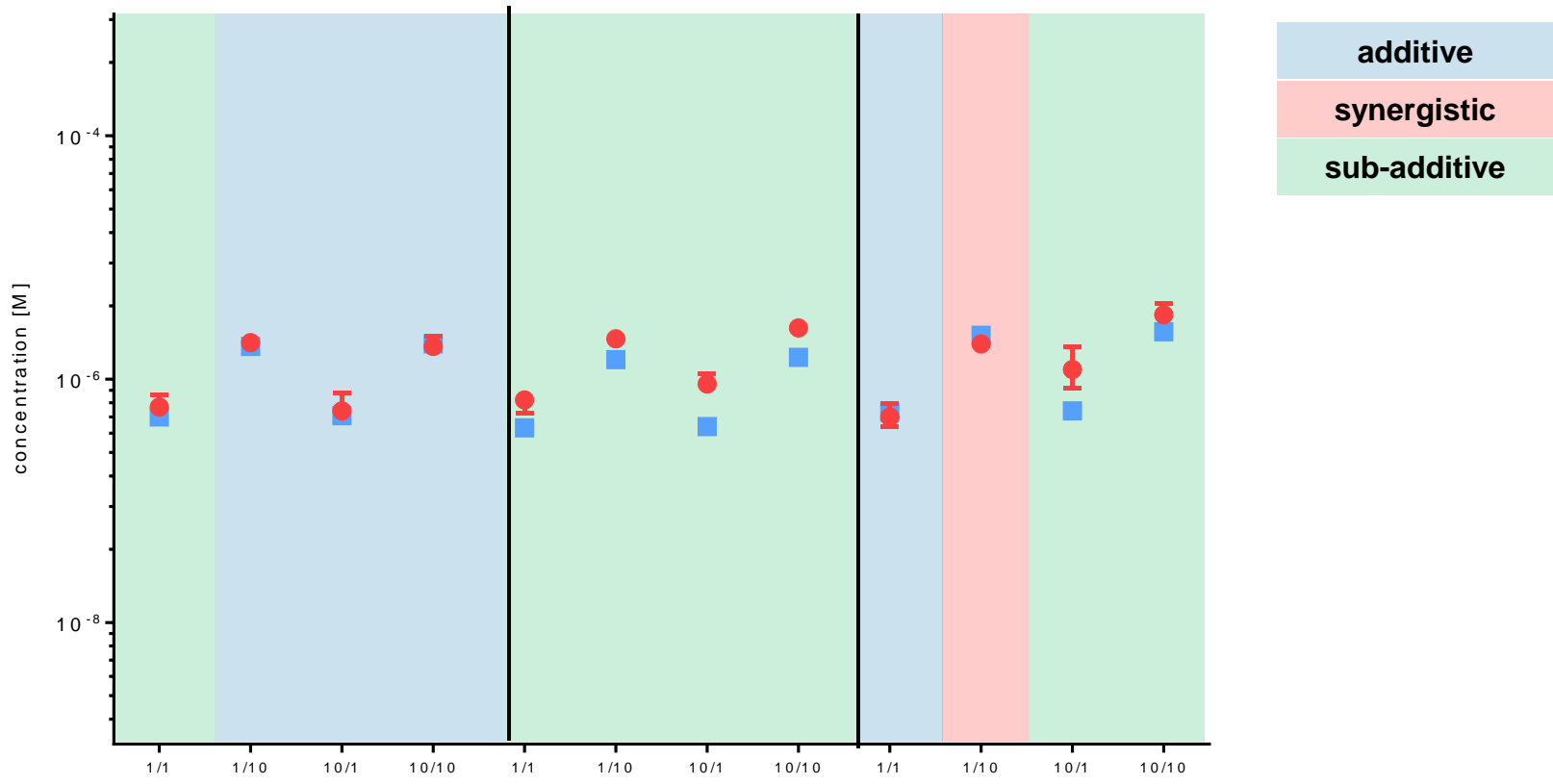
EC01 mix – EC01
EC01 mix – EC10
EC10 mix – EC01
EC10 mix – EC10

fludioxonil
fenhexamid

ER α CALUX

propamocarb
fludioxonil
fenhexamid

chlorpyrifos
fludioxonil
fenhexamid



additive
synergistic
sub-additive

statistical deviations from additivity are due to narrow 95% CIs → not of biological relevance
additive effects in low effect concentrations of estrogenic pesticides in the ER α CALUX

Estrogenic mixtures - hER α

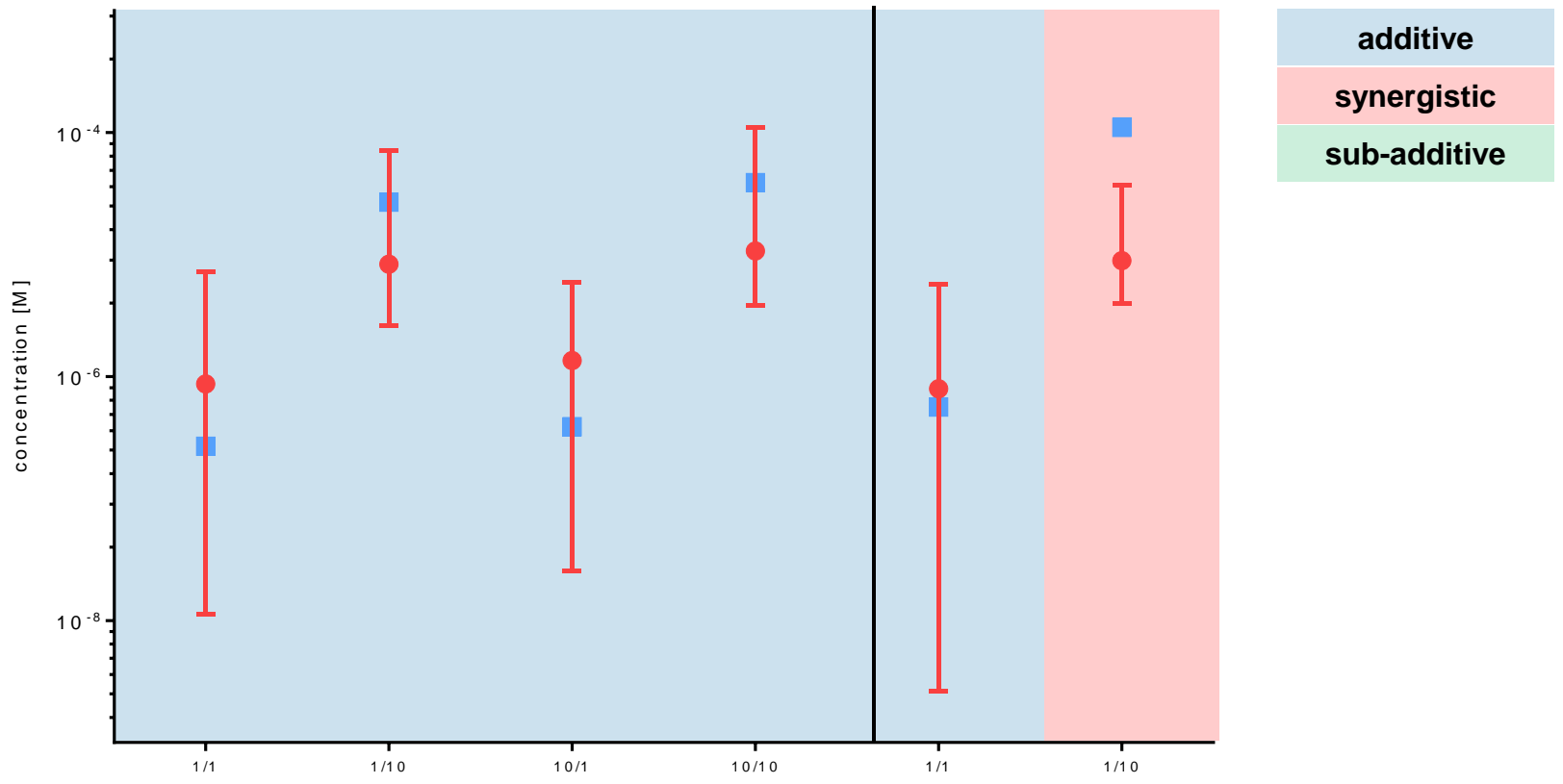
1/1
1/10
10/1
10/10

EC01 mix – EC01
EC01 mix – EC10
EC10 mix – EC01
EC10 mix – EC10

YES

fludioxonil
fenhexamid

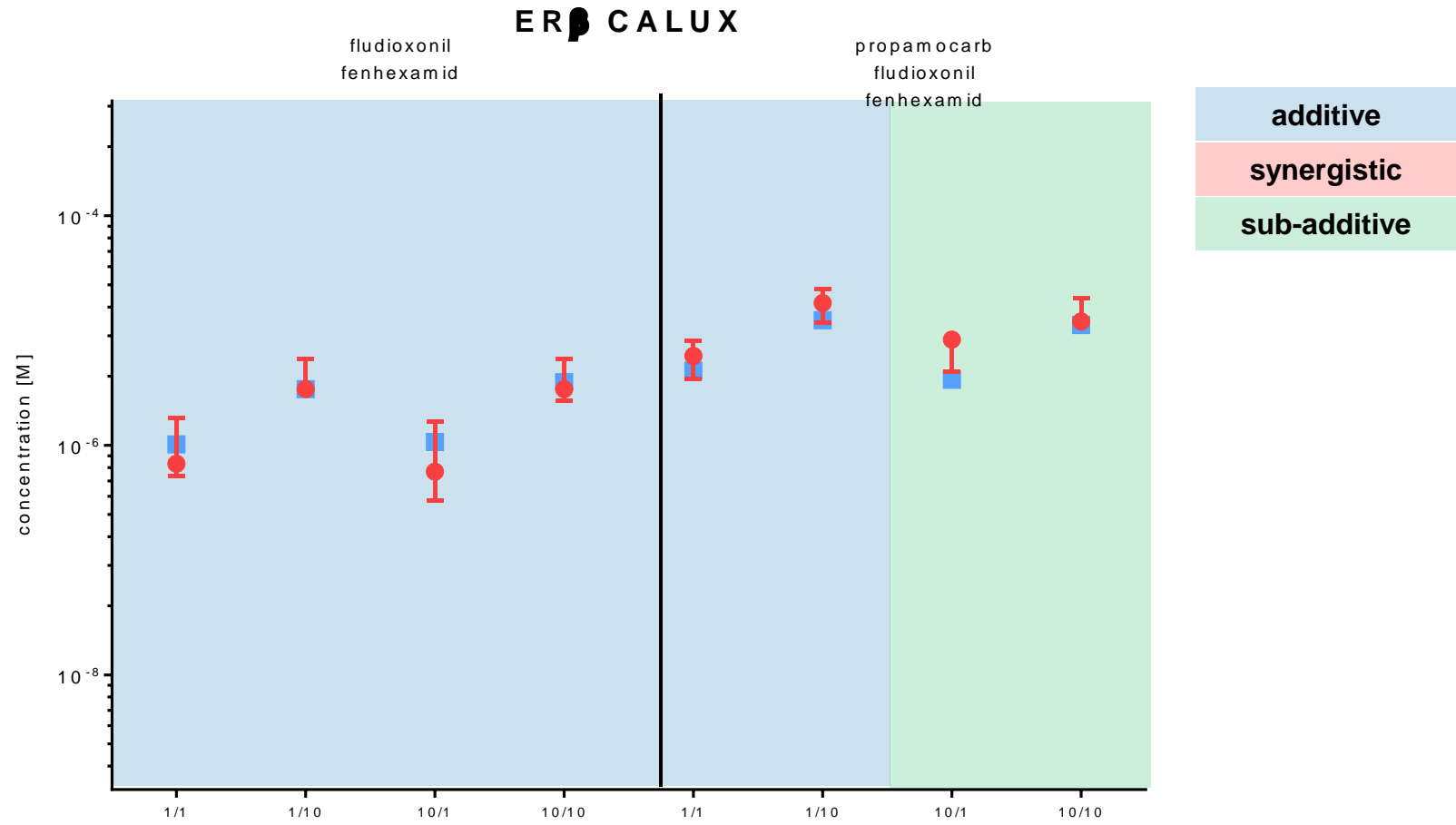
chlorpyrifos
fludioxonil
fenhexamid



additive
synergistic
sub-additive

additive effects in low effect concentrations of estrogenic pesticides in the YES

Estrogenic mixtures - hERβ



additive effects in low effect concentrations of estrogenic pesticides in the ERβ CALUX

Anti-androgenic mixtures

1/1
1/10
10/1
10/10

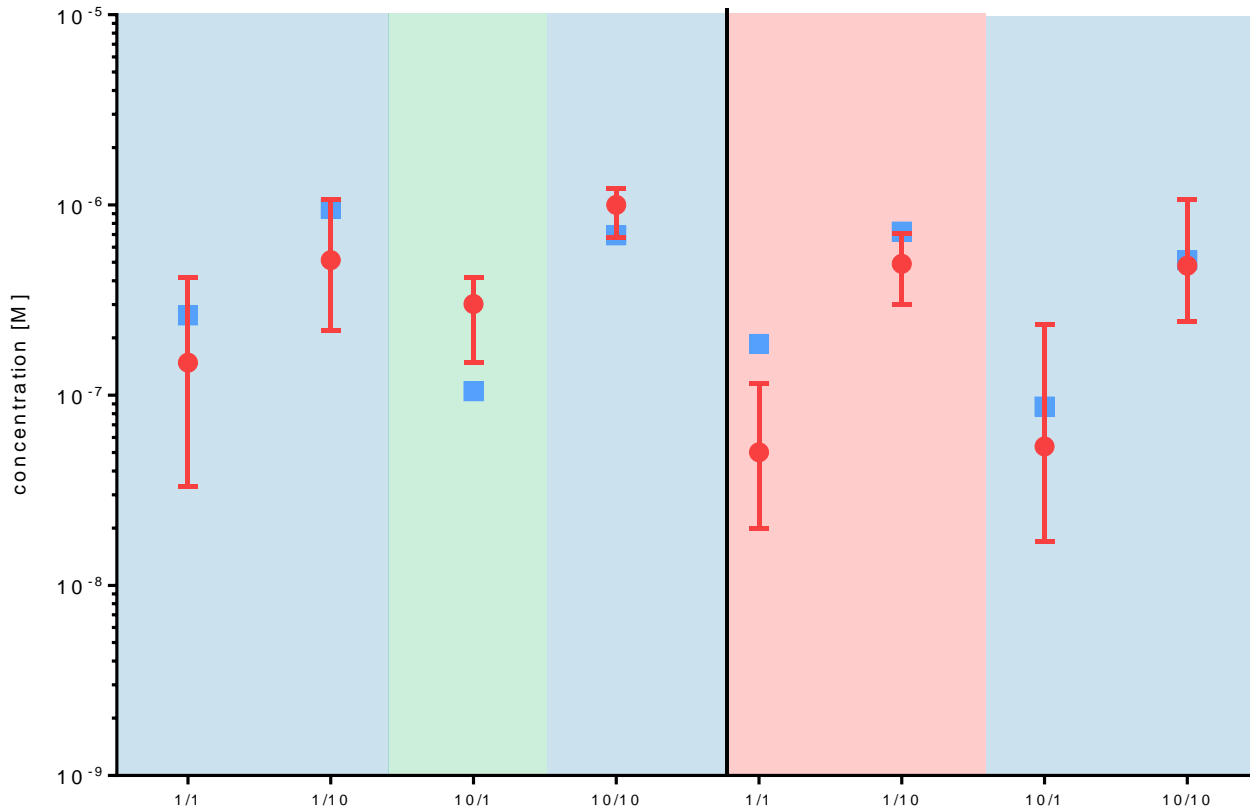
IC01 mix – IC01
IC01 mix – IC10
IC10 mix – IC01
IC10 mix – IC10

YAS

procymidone
vinclozolin

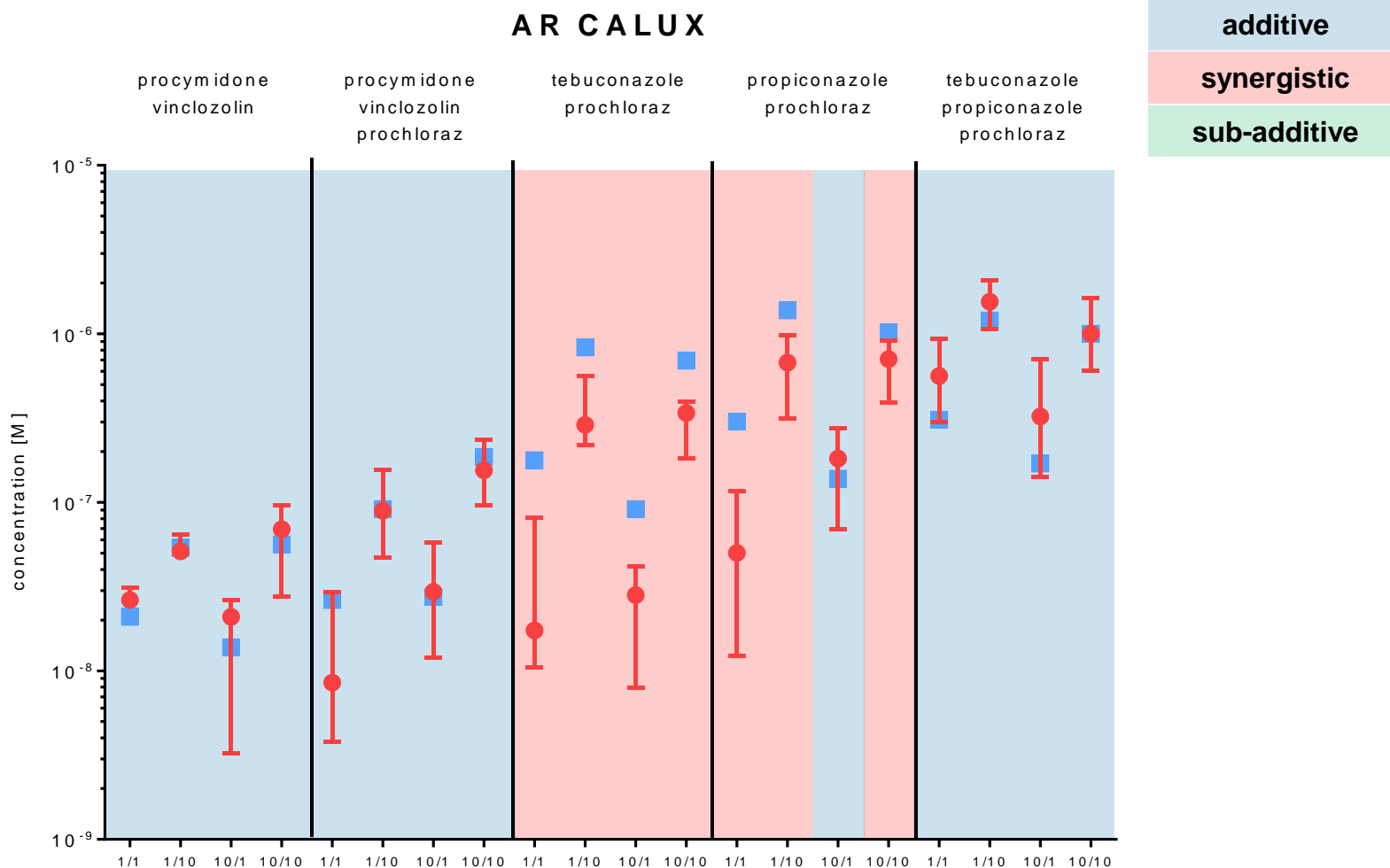
procymidone
vinclozolin
prochloraz

additive
synergistic
sub-additive



additive effects in low inhibitory concentrations of anti-androgenic fungicides in the YAS

Anti-androgenic mixtures



additive effects in low inhibitory concentrations of anti-androgenic fungicides in the AR CALUX

Conclusion: Estrogenic and anti-androgenic pesticides

identification of estrogenic and anti-androgenic effects ✓

additive mixture effects ✓

- **additive effects** of estrogenic at the hER α and the hER β and anti-androgenic pesticides at the hAR

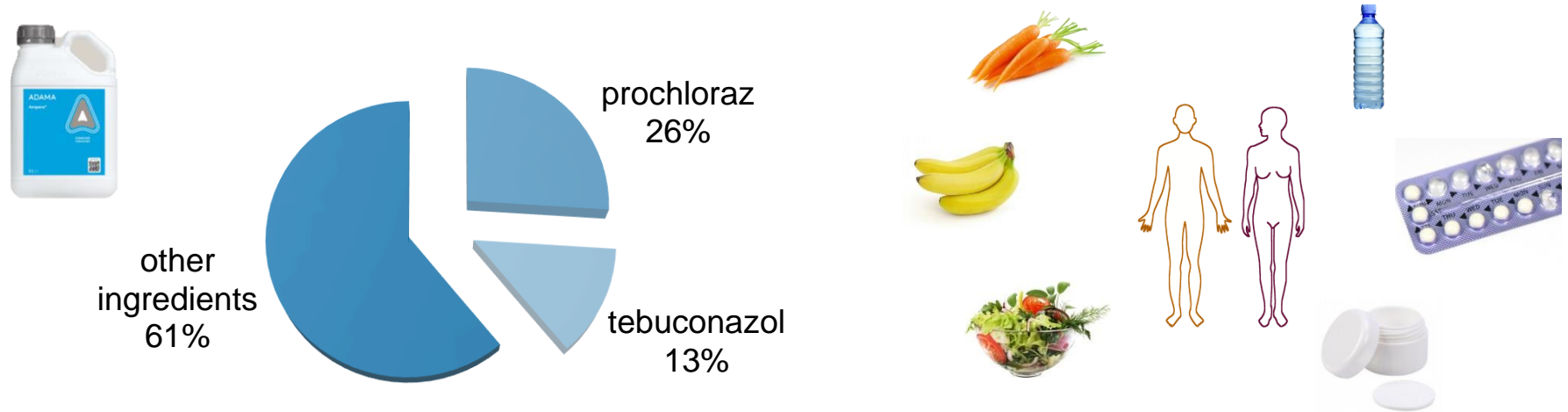
identification of mechanistic data and **additive behavior** of the tested pesticides at the respective receptors

Assumptions concerning cumulative risk assessment

- All used test systems were suitable for the **screening** of estrogenic and anti-androgenic pesticides
- **additivity** of low effect/inhibitory concentrations could be shown in all used test systems
- **CA** is a suitable mathematical model for estrogenic and anti-androgenic pesticide mixtures
- **Hazard identification in a tiered risk assessment approach**

hormonally active pesticides → cumulative risk assessment groups?

- pesticides sharing a mode of action ✓
- **BUT** what about other ingredients present in pesticide formulations, as well as many other anti-androgenic and estrogenic chemicals?
- → **risk assessment for more complex chemical mixtures**



Acknowledgement

Thank you for your attention!

Special thanks
to
**Prof. Pablo
Steinberg**
and my
colleagues
at the **Institute
for Food
Toxicology**



**Prof. Frank Klawonn
Boris Nguema Bekale**



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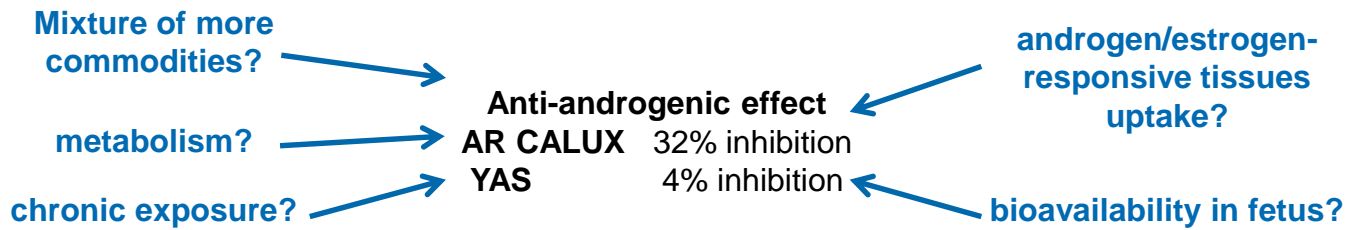
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<http://forthemommas.com/wp-content/uploads/2015/10/Lettuce.jpg>

“Real life” worst case scenario – relevance for humans



Max. residue found in 2013 for procymidone on lettuce: 3.7 mg/kg	person with 70 kg body weight ~ 5 L blood	25% processing factor 231 µg/250 g lettuce
maximum uptake (80%): 37 µg/L blood	blood concentration 0.13 µM	
97% in a mixture		

Max. residue found in 2013 for vinclozolin on lettuce: 0.121 mg/kg	person with 70 kg body weight ~ 5 L blood	25% processing factor 8 µg/250 g lettuce
maximum uptake (85%): 1 µg/L blood	blood concentration 4.5 nM	
3% in a mixture		

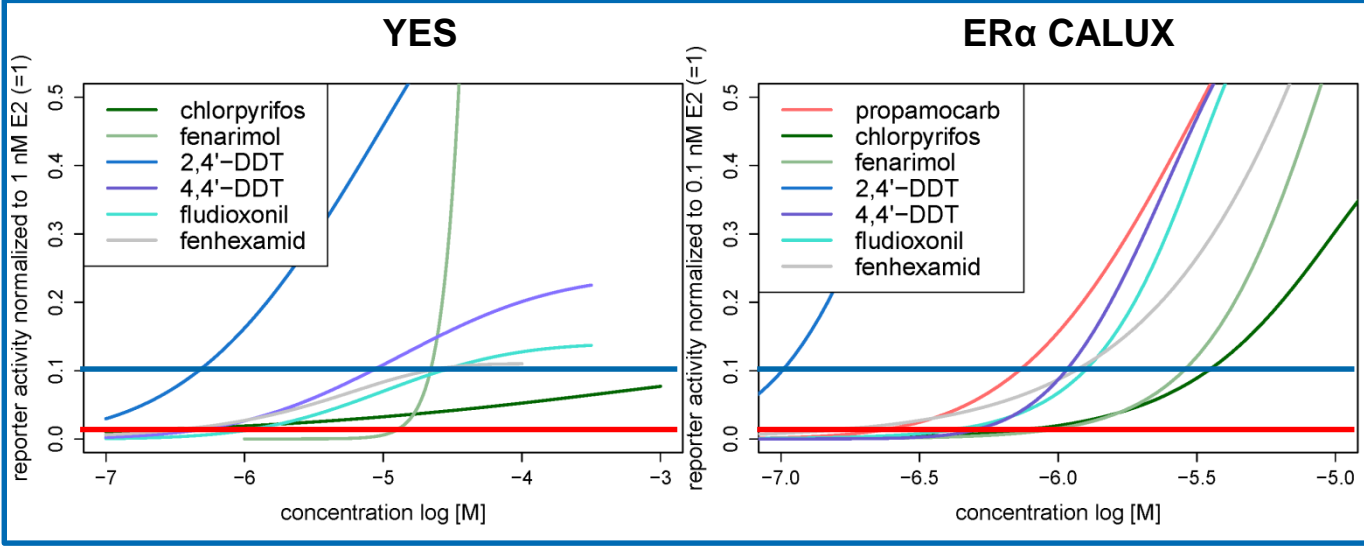


Estrogenic substances

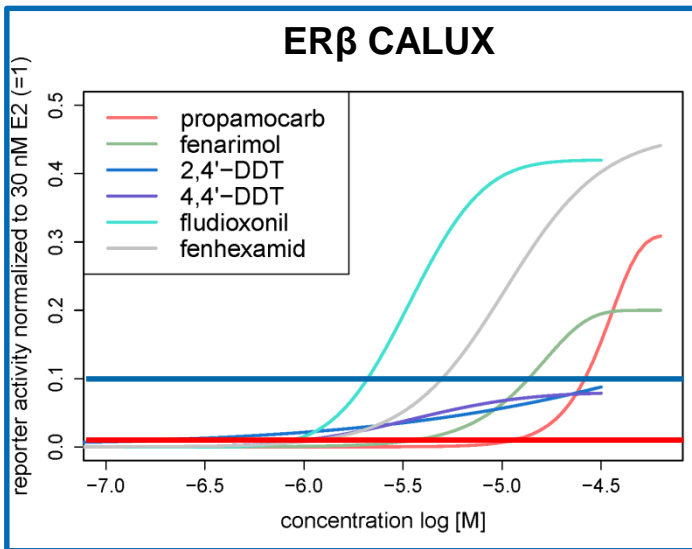
hER α

EC10

EC01



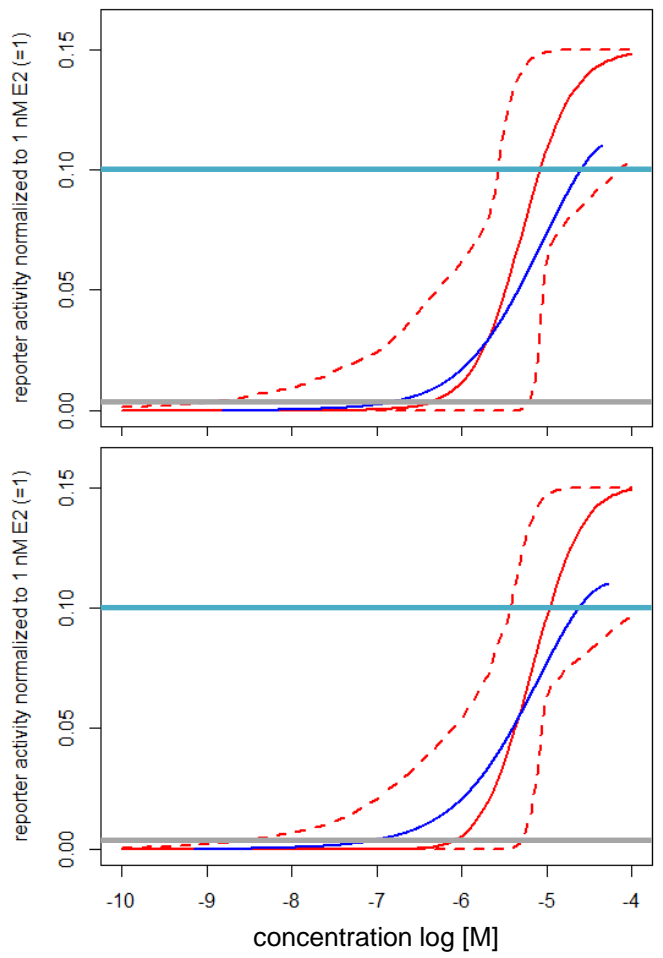
hER β



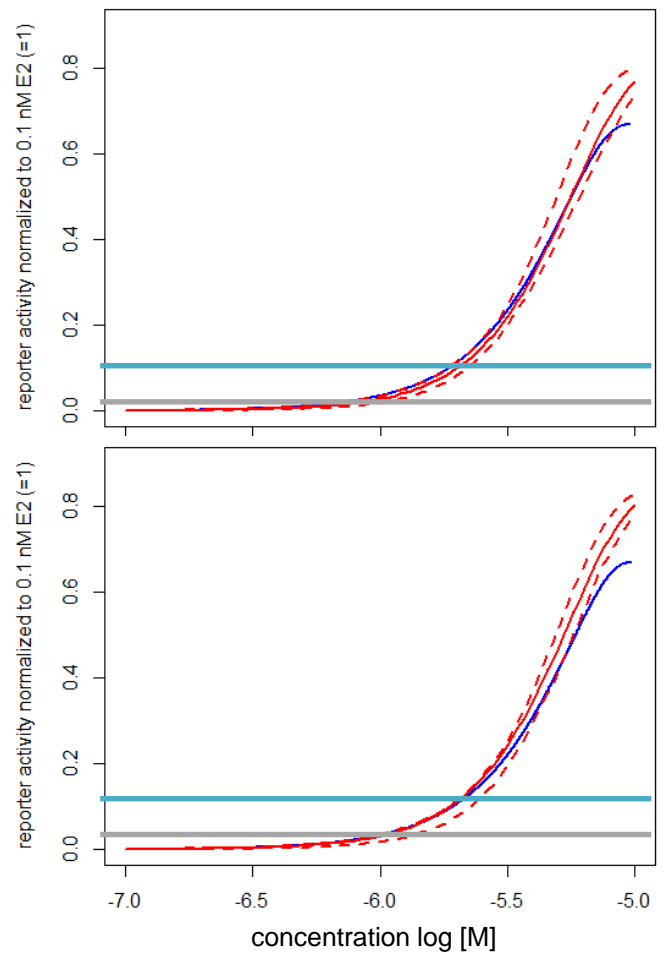
Estrogenic mixtures - hER α

YES **fludioxonil & fenhexamid** **ER α CALUX**

EC10
EC01



EC01
Mix



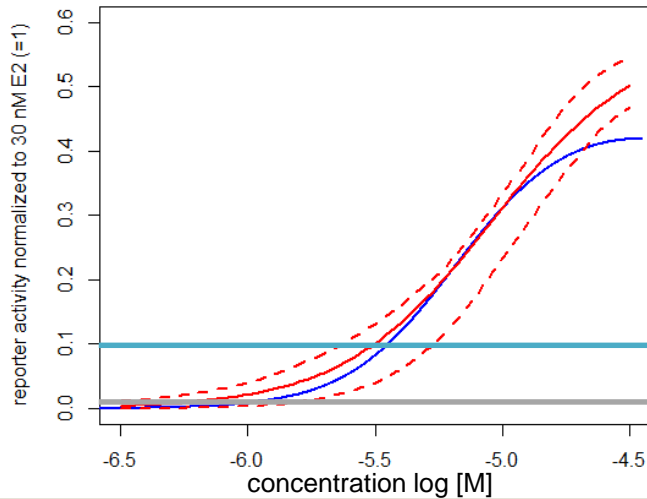
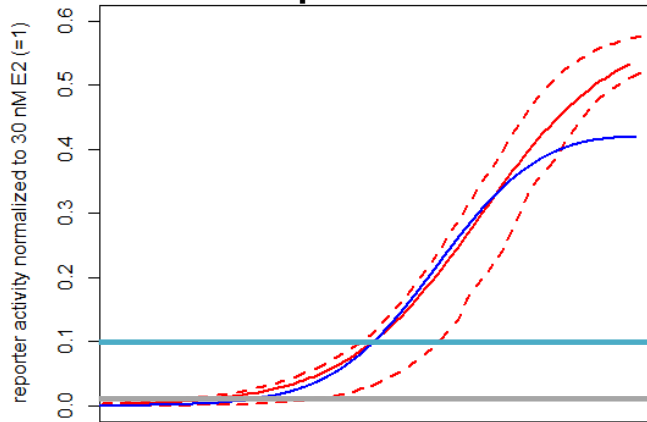
EC10
Mix

additive effects of fludioxonil and fenhexamid in YES and ER α CALUX

Estrogenic mixtures - hER β

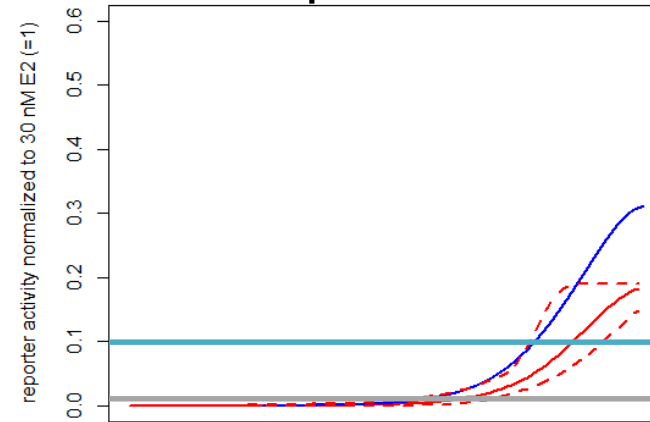
**fludioxonil & fenhexamid
ER β CALUX**

EC10
EC01

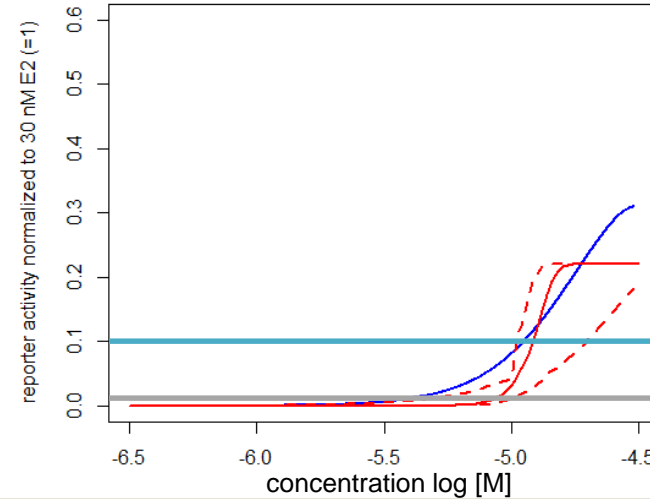


**fludioxonil, fenhexamid & propamocarb
ER β CALUX**

EC01
mix

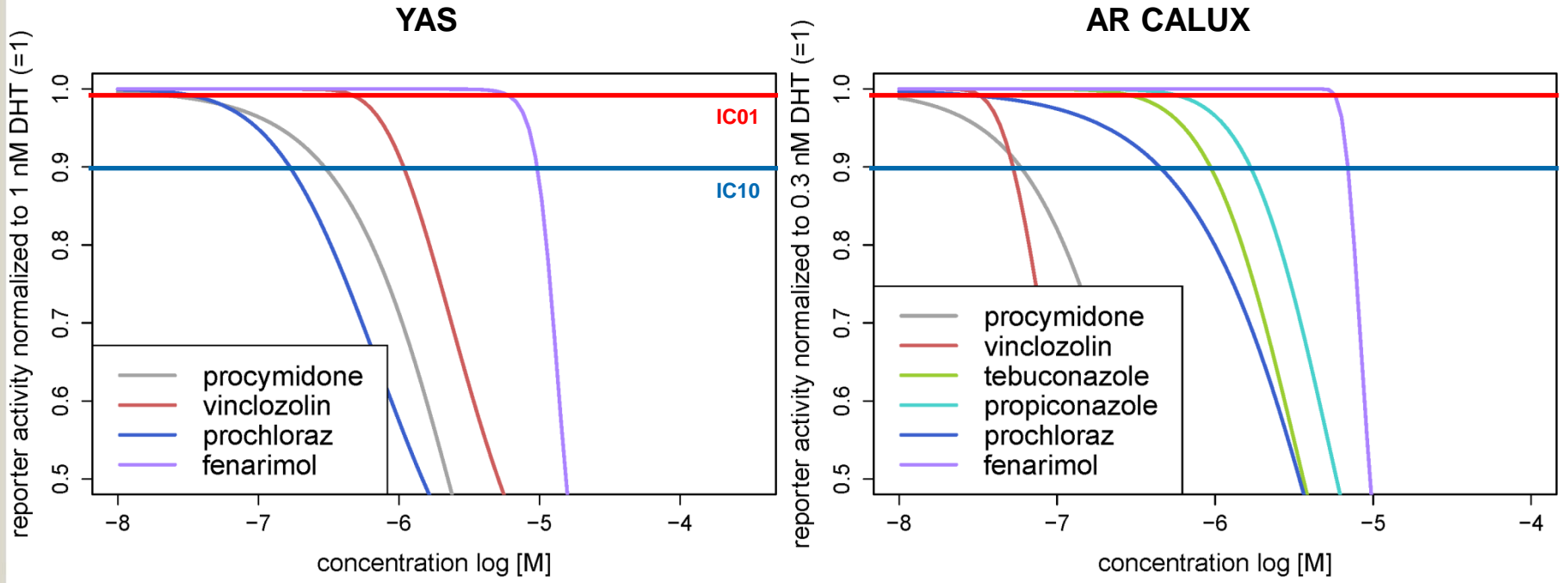


EC10
mix



additive effects in ER β CALUX

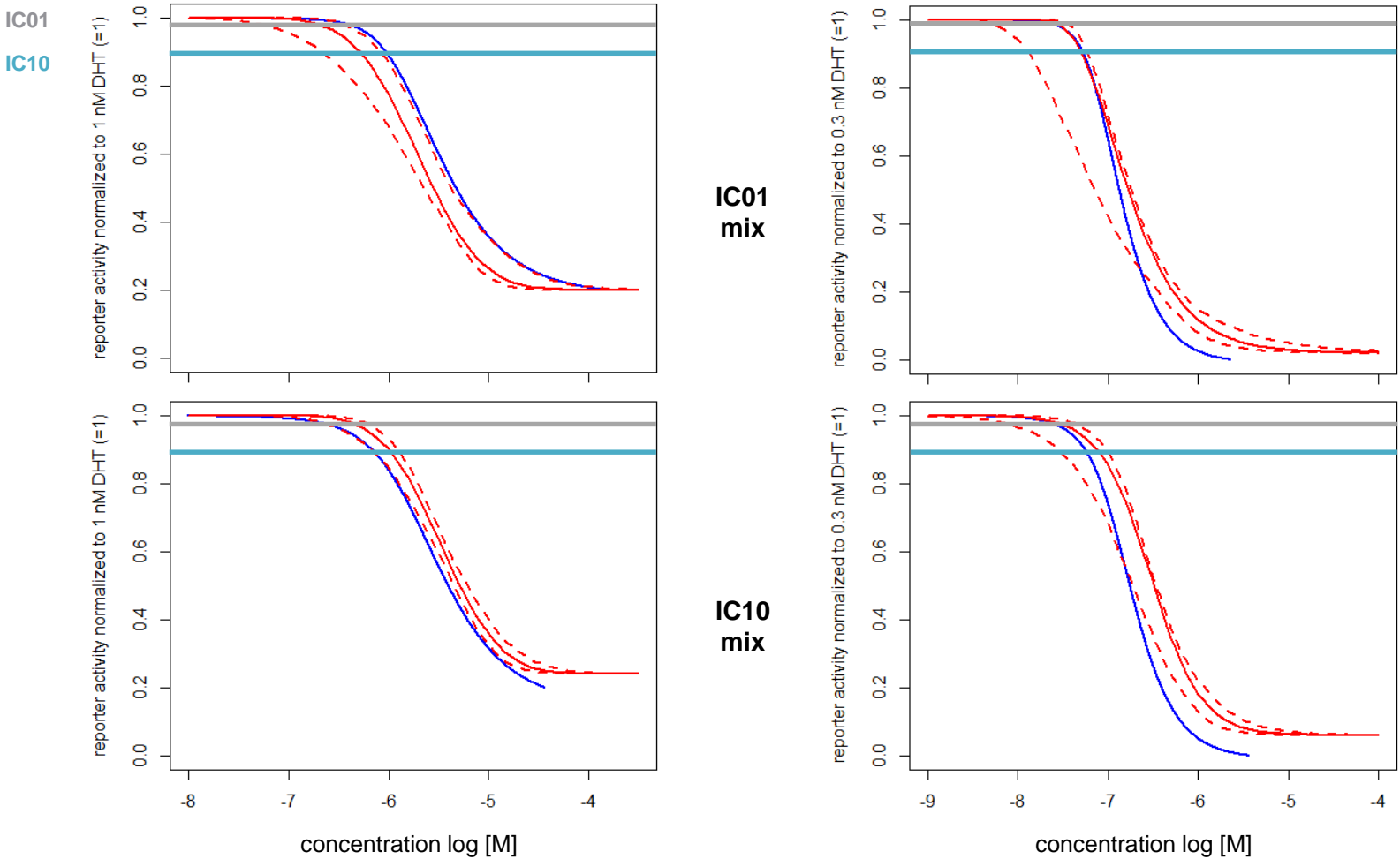
Anti-androgenic substances



The triazoles (tebuconazole and propiconazole) inhibited cell growth of the yeast cells at concentrations $\geq 0.1 \mu\text{M}$ and could not be identified as anti-androgenic substances in the YAS.

Anti-androgenic mixtures

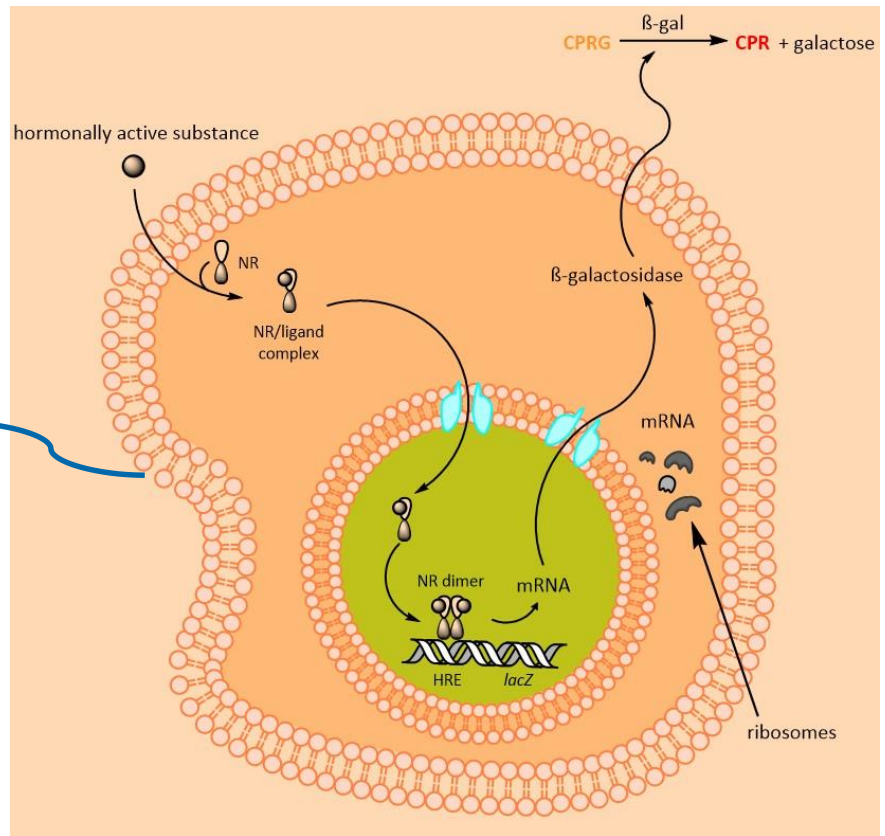
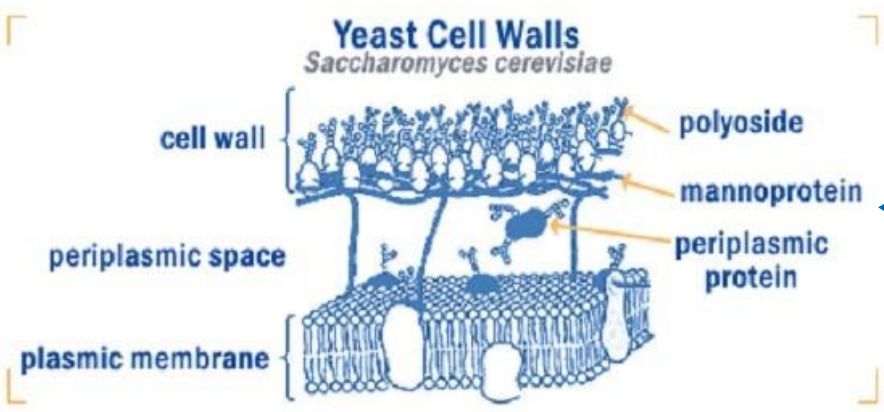
YAS procymidone & vinclozolin AR CALUX



Test systems

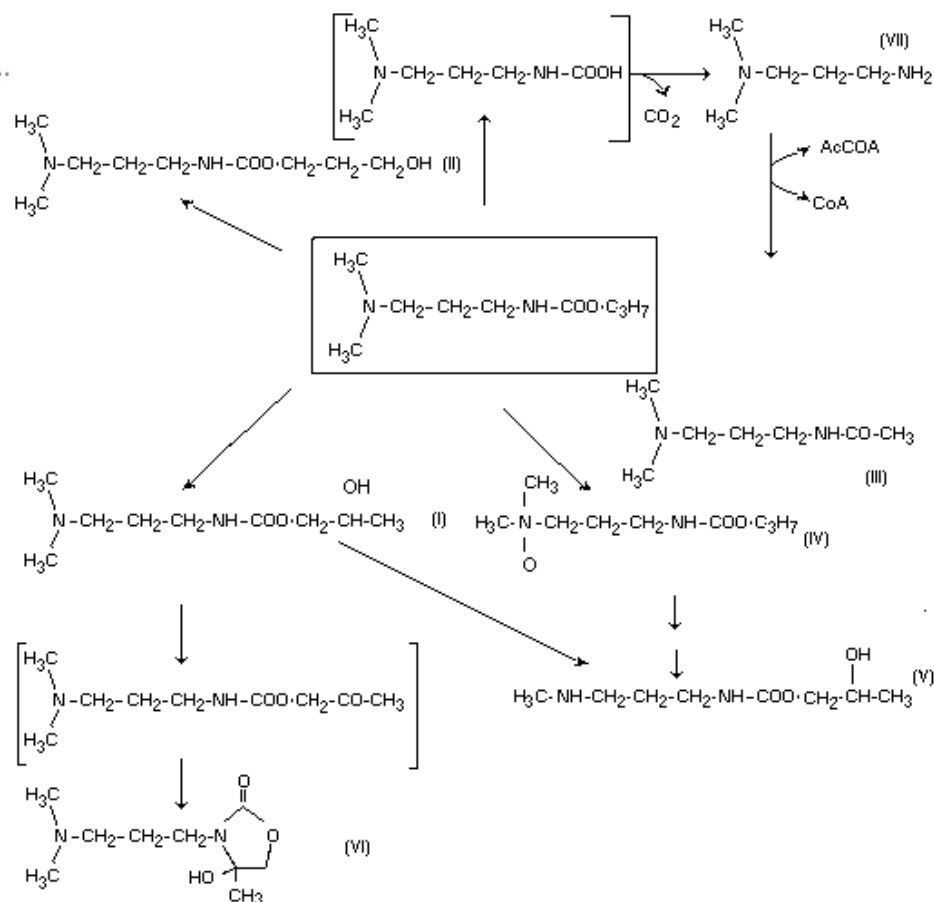
YAS/YES

Yeast-based Androgen/Estrogen Screen



Used according to protocols of BASF
(n=≥5 with quadruplicates in 96 well plates)

FIGURE 1. PROPOSED METABOLIC PATHWAYS FOR PROPAMOCARB IN THE RAT



- I: 2 - hydroxypropyl ester propamocarb
- II: 3 - hydroxypropyl ester propamocarb
- III: N - (3 - dimethyl - aminopropyl) - acetamide
- IV: propamocarb N- oxide
- V: 3 - (3 - dimethylaminopropyl) - 4 - hydroxy - 4 - methyl - oxazolidin - 2 - one
- VII: 3 - (N, N - dimethylamino) - propylamine

Mechanisms of the pesticides

Anti-androgenically acting substances

pesticide	chem. group	mechanism	
procymidone	dicarboximide	fungicide	osmotic signal transduction → MAP/histidine kinase
vinclozolin	dicarboximide	fungicide	osmotic signal transduction → MAP/histidine kinase
tebuconazole	triazole	fungicide	sterol biosynthesis in membranes → C14-demethylase
propiconazole	triazole	fungicide	sterol biosynthesis in membranes → C14-demethylase
fenarimol	pyrimidine	fungicide	sterol biosynthesis in membranes → C14-demethylase
prochloraz	imidazole	fungicide	sterol biosynthesis in membranes → C14-demethylase

Mechanisms of the pesticides

Estrogenically acting substances

pesticide	chem. group	mechanism	
fenarimol	pyrimidine	fungicide	sterol biosynthesis in membranes → C14-demethylase
fenhexamid	hydroxyanilide	fungicide	sterol biosynthesis in membranes → 3-Ketoreductase in C4-demethylation
fludioxonil	phenylpyrrole	fungicide	osmotic signal transduction → MAP/histidine kinase
propamocarb	carbamate	fungicide	lipid synthesis and membrane integrity → cell membrane integrity, fatty acids
pirimicarb	carbamate	insecticide	CNS → inhibition of acetylcholinesterase
chlorpyrifos	organophosphate	insecticide	CNS → inhibition of acetylcholinesterase
4,4'-DDT	organochloride	insecticide	CNS → Na²⁺ channels are opened
2,4-DDT	organochloride	insecticide	CNS → Na²⁺ channels are opened

Mixture ratios: estrogenic pesticides

test system	compounds	EC01	EC10
ER α CALUX	fenhexamid fludioxonil	62.81% 37.19%	67.61% 32.39%
YES	fenhexamid fludioxonil	20.84% 79.16%	44.86% 55.14%
ER α CALUX	fenhexamid fludioxonil chlorpyrifos	38.48% 22.79% 38.73%	35,73% 17.12% 47.15%
YES	fenhexamid fludioxonil chlorpyrifos	19.37% 73.60% 7.02%	-
ER α CALUX	fenhexamid fludioxonil propamocarb	51.73% 30.63% 17.64%	57.00% 27.31% 15.69%
ER α CALUX	fenhexamid fludioxonil	62.81% 37.19%	67.61% 32.39%
YES	fenhexamid fludioxonil	20.84% 79.16%	44.86% 55.14%
ER β CALUX	fenhexamid fludioxonil	56.77% 43.23%	70.56% 29.44%
ER β CALUX	fenhexamid fludioxonil propamocarb	8.63% 6.57% 84.80%	15% 6.26% 78.74%

Mixture ratios: anti-androgenic pesticides

test system	compounds	EC01	EC10
AR CALUX	procymidone vinclozolin	21.60% 78.40%	52.87% 47.13%
YAS	procymidone vinclozolin	5.20% 94.80%	21.61% 78.39%
AR CALUX	procymidone vinclozolin prochloraz	11.48% 41.68% 46.84%	10.55% 9.40% 80.05%
YAS	procymidone vinclozolin prochloraz	4.86% 88.56% 6.58%	19.27% 69.89% 10.84%
AR CALUX	tebuconazole prochloraz	89.47% 10.53%	67.61% 32.39%
AR CALUX	propiconazole prochloraz	94.31% 5.69%	78.79% 21.21%
AR CALUX	propiconazole tebuconazole prochloraz	63.60% 32.57% 3.83%	54.61% 30.69% 14.70%
AR CALUX	procymidone vinclozolin	21.60% 78.40%	52.87% 47.13%
YAS	procymidone vinclozolin	5.20% 94.80%	21.61% 78.39%