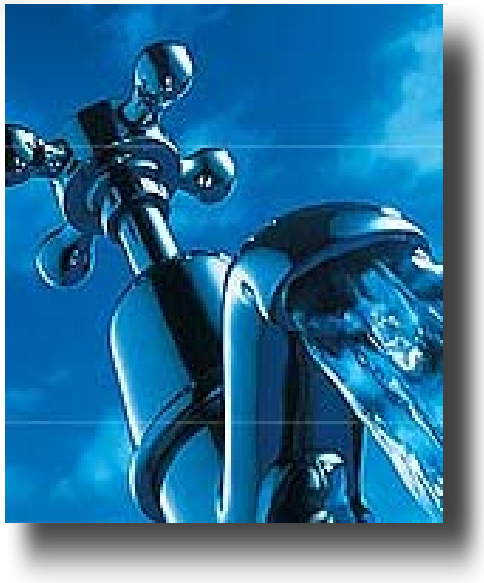
International Symposium for HPTLC
11. – 13. June 2008, Helsinki

Zweckverband
Landeswasserversorgung

HPTLC/AMD with *Vibrio fischeri* as an example for bioactivity-based detection – A new dimension in analytics



Wolfgang Schulz, Wolfram Seitz, and Walter H. Weber

Zweckverband Landeswasserversorgung,
Langenau, Germany

Structure

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- Introduction
- *Vibrio fischeri* HPTLC test /
  Calculation of inhibition profiles
- Aspects of the luminescence inhibition test /
  Application examples
- Advanced data evaluation
- Conclusions

Landeswasserversorgung is situated in South Germany

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Distribution area of LW in South West Germany

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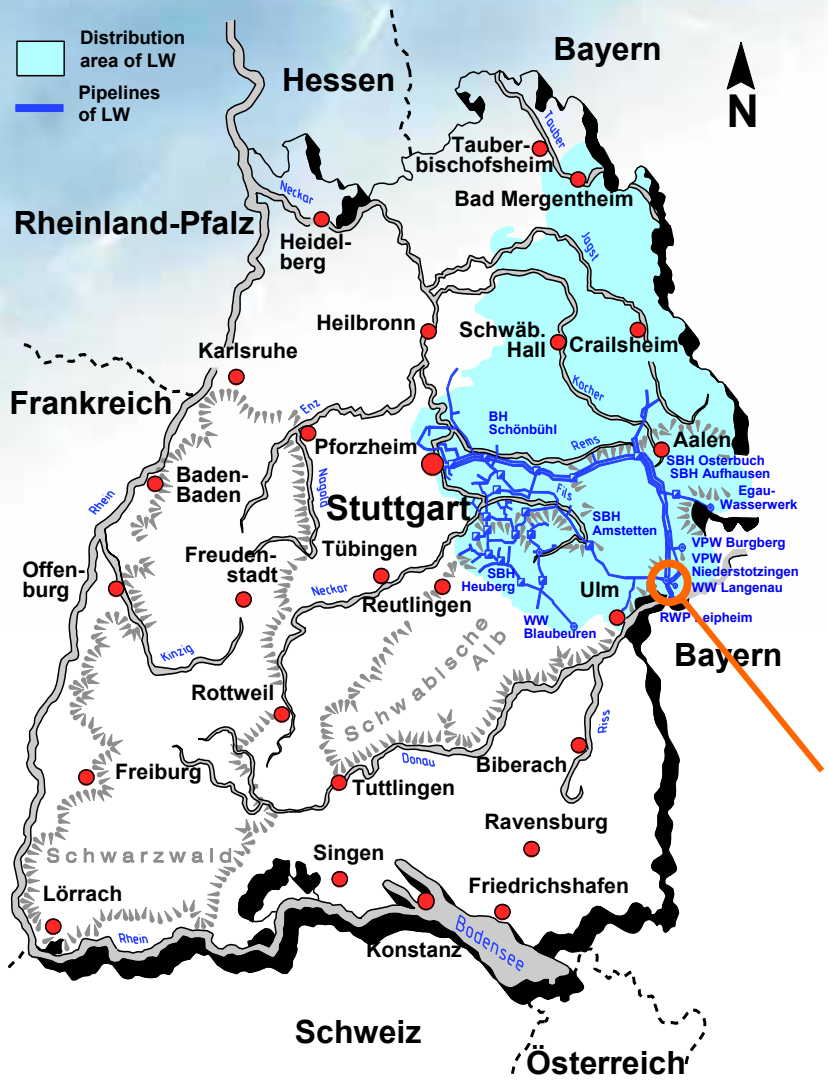

- Distribution area in South West Germany
- 3 Million customers within distribution area
- Long-distance water fraction approx. 50%

Langenau Waterworks

Use of different resources for drinking water production

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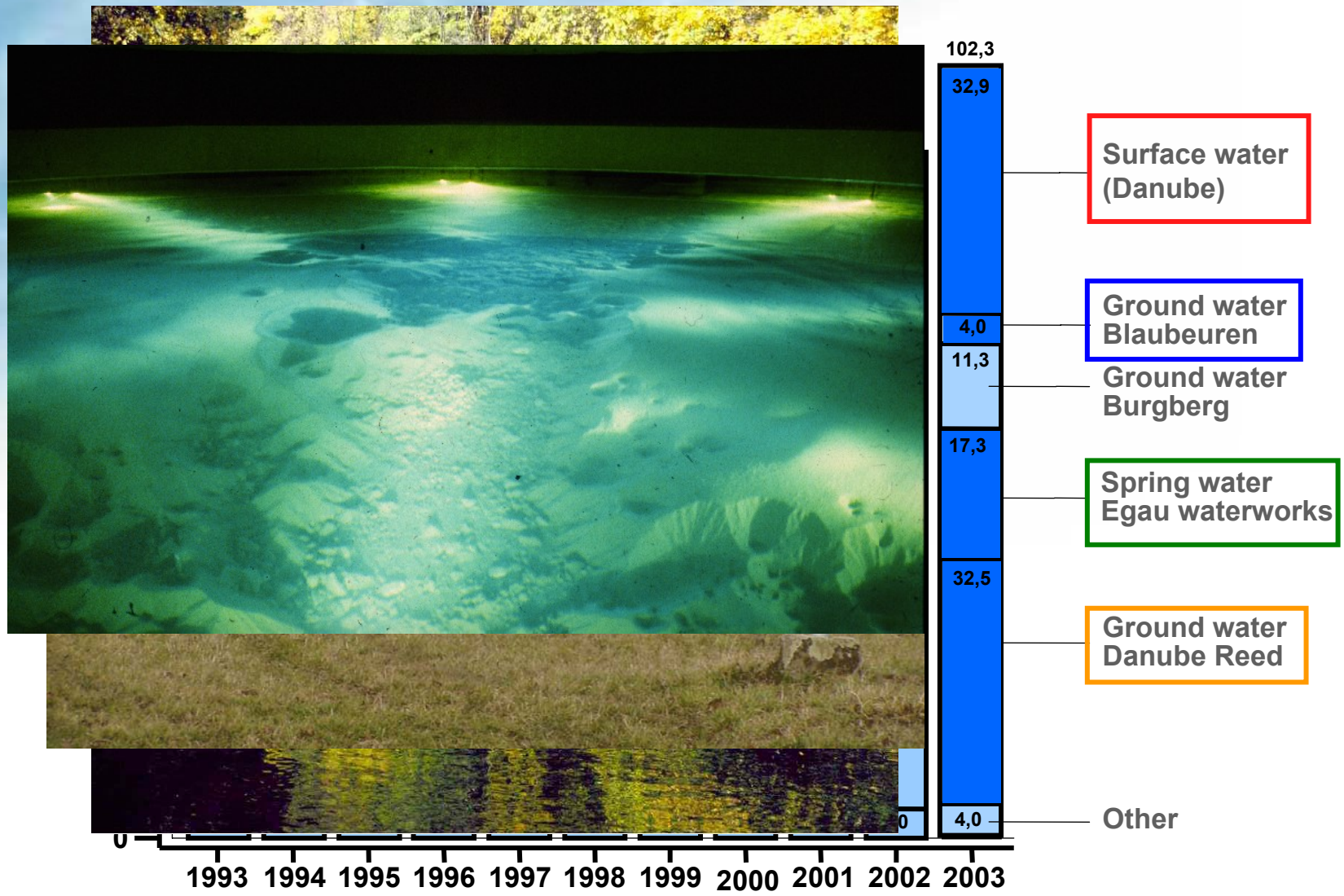

River Danube in South Germany (Leipheim)

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Direct abstraction of river water (near Leipheim)

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Concept of „Multidimensional Screening“ for water contaminants

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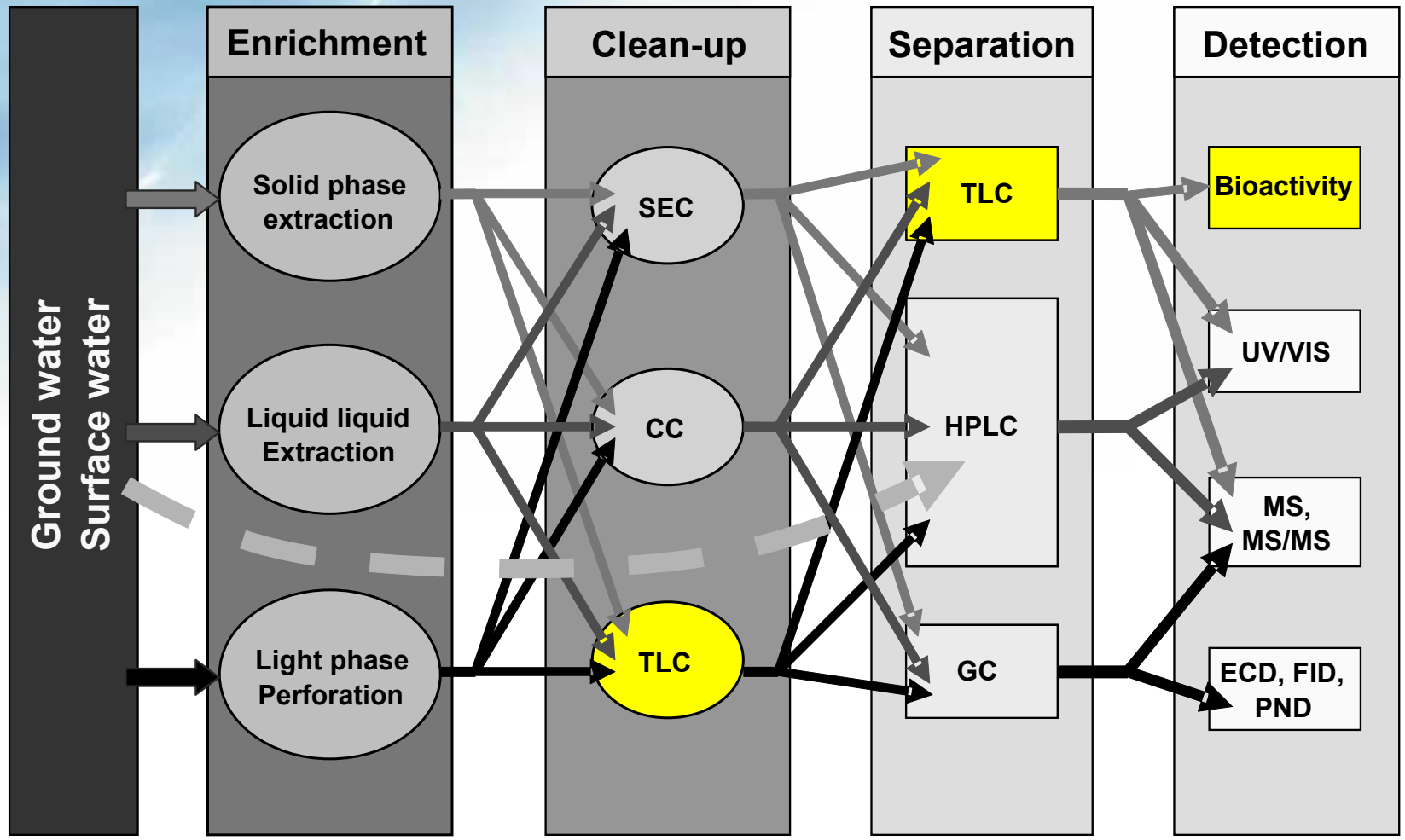

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Selected bioactivity-based tests for HPTLC

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Test	Purpose
Vibrio fischeri (luminescent bacterium)	Screening test for bioactivity
Bacillus subtilis (bacterium)	Cytotoxicity test for antibiotic or cytostatic substances
Rhodoturula rubra (yeast)	Cytotoxicity test for fungicides
Acetylcholinesterase (enzyme)	Neurotoxicity test

Reference: C. Weins (2006), Dissertation, University of Basel, Switzerland

Luminescent bacteria *Vibrio fischeri*

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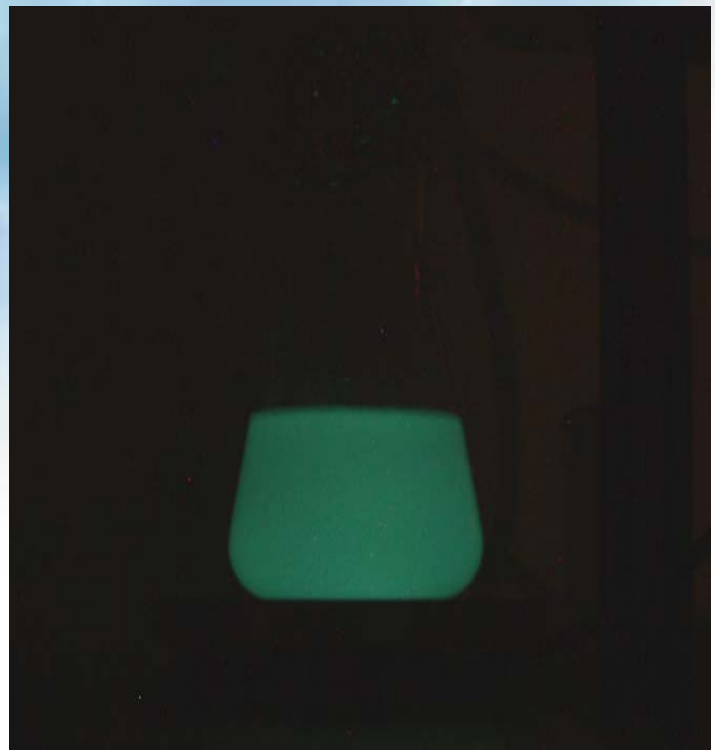
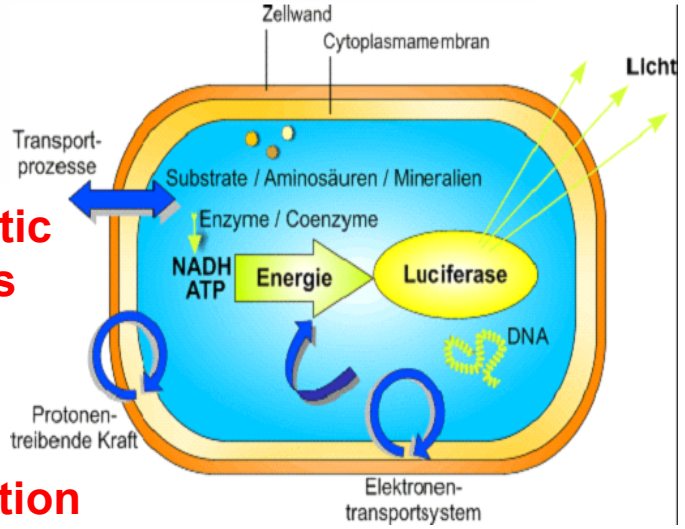


Suspension of *Vibrio fischeri* in water

- Marine bacterium
- Lives in symbiosis with marine life forms
- Continuous bioluminescence
- Bioluminescence is coupled to energy metabolism



Narcotic effects

Isolation

Inhibition of electron transport

Reference: Reinecke, N. (2003), Dissertation, Universität Hamburg

Vibrio fischeri in water analysis

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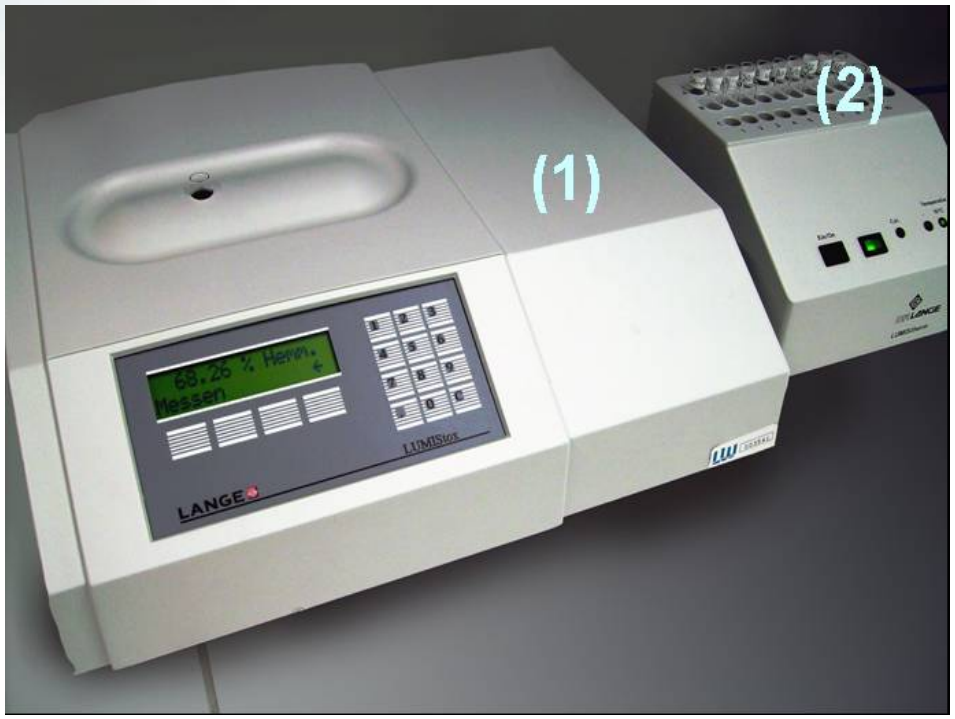
- **Classic application: Cuvette test (DIN 11348)**

  → **Detection of combination effects of toxic compounds (synergistic effects)**



(1) LUMIStox 300
(2) LUMIStherm

Luminescence inhibition test on TLC plates

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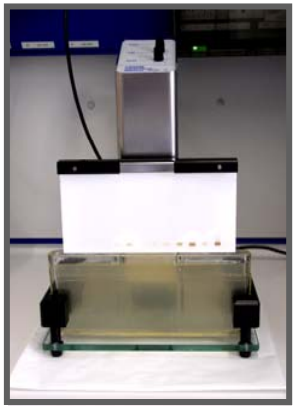
Sample (extract)

Application (TLC Sampler)

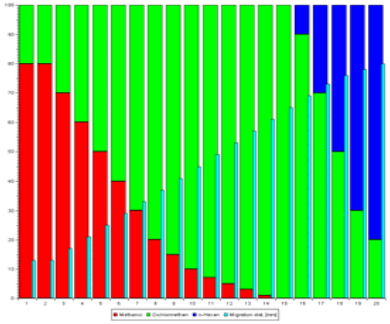
Development

Immersion



Reference:
Weisemann, C., Kreiss, W., Rast, H-G., Eberz. G.; "Analytical Method for Investigating Mixtures for Toxic Components." European Patent No: EP 0 588 139 B1.

Test Kit:
Bioluminex™, ChromaDex, CA



Detection of luminescence

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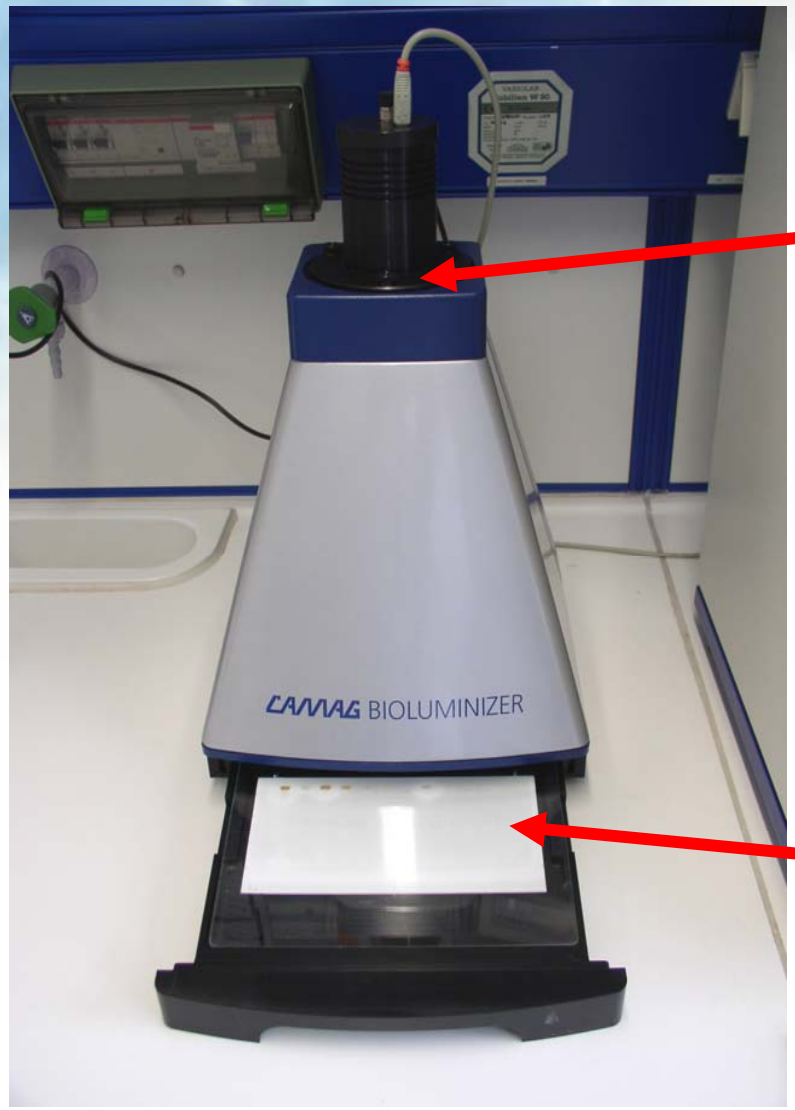

CCD camera
Camag Bioluminizer
(typical detection
time 40 sec)

TLC plate
after immersion
into bacteria suspension

Data evaluation –
Calculation of inhibition profiles

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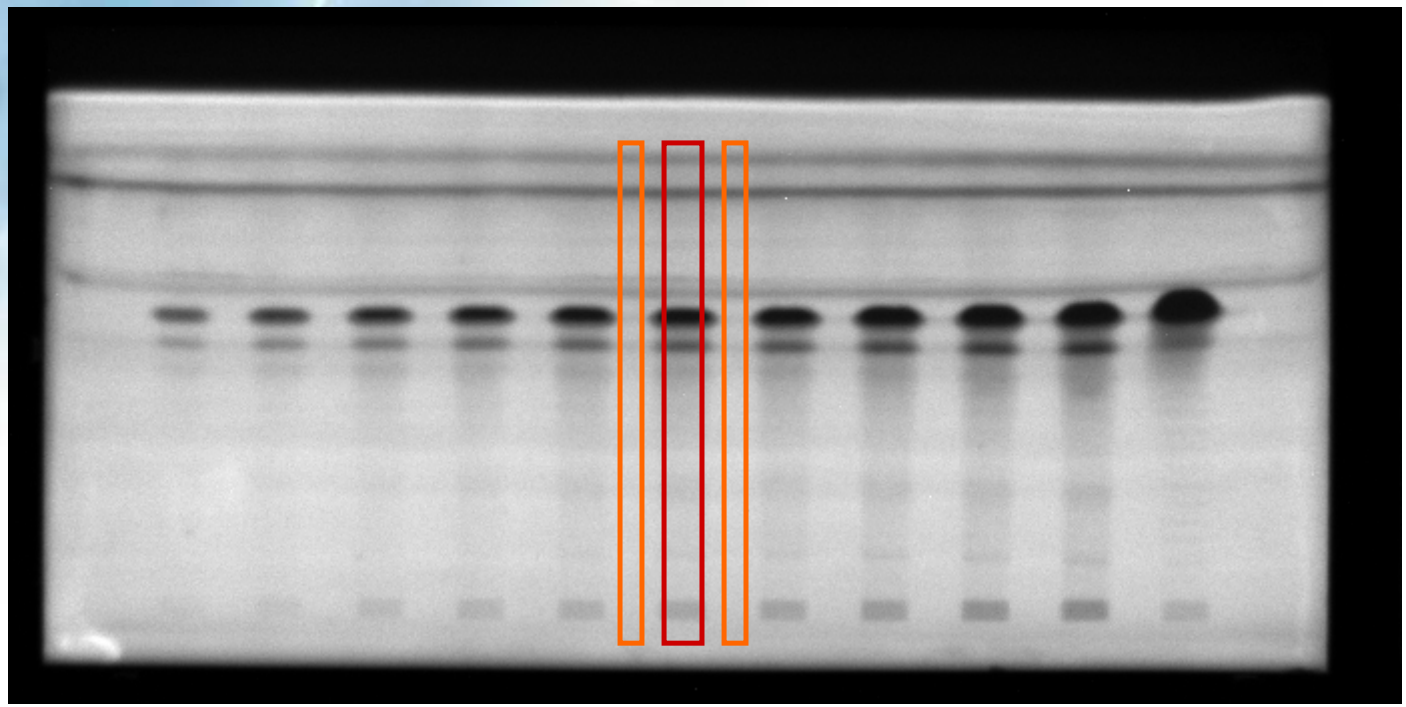

Sample volume [µL]	10	20	30	40	50	60	70	80	90	100	Reference

Calculation of inhibition profiles

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Nr.	UGl	Track6	UGr	MW UG	Inhibition [%]
1	192,87	191,72	187,5	190,19	-0,81
2	193,1	191,97	188,35	190,73	-0,65
3	192,05	191,72	188,65	190,35	-0,72
4	195,05	192,47	190,75	192,90	0,22
5	196,15	192,69	192,1	194,13	0,74
6	197,8	189,25	193,7	195,75	3,32
7	197,05	184,14	193,65	195,35	5,74
8	198,2	177,42	193,7	195,95	9,46
9	199,4	171,47	195,35	197,38	13,12
10	199	168,53	195,95	197,48	14,66
11	199,25	167,58	196,65	197,95	15,34
12	200,25	166,64	197,6	198,93	16,23
13	201,75	168,14	199,35	200,55	16,16
⋮					⋮
233	159,35	153,31	154,55	156,95	2,32
234	153,15	144,92	146,45	149,80	3,26
235	144,05	137,28	139,55	141,80	3,19
236	137,75	132,97	134,9	136,33	2,46
237	136,45	133,89	133,35	134,90	0,75
238	139,35	137,83	136,95	138,15	0,23
239	146,4	146,19	146,1	146,25	0,04
240	154,75	154,36	155,8	155,28	0,59
241	162,5	162,92	163,8	163,15	0,14
242	169,95	170,97	171,55	170,75	-0,13

$$I_n^P = 1 - \frac{i_n^S}{i_n^R}$$

I = Inhibition
i = Light intensity
S = Sample
R = Reference
n = Number of pixel

Inhibition chromatogram

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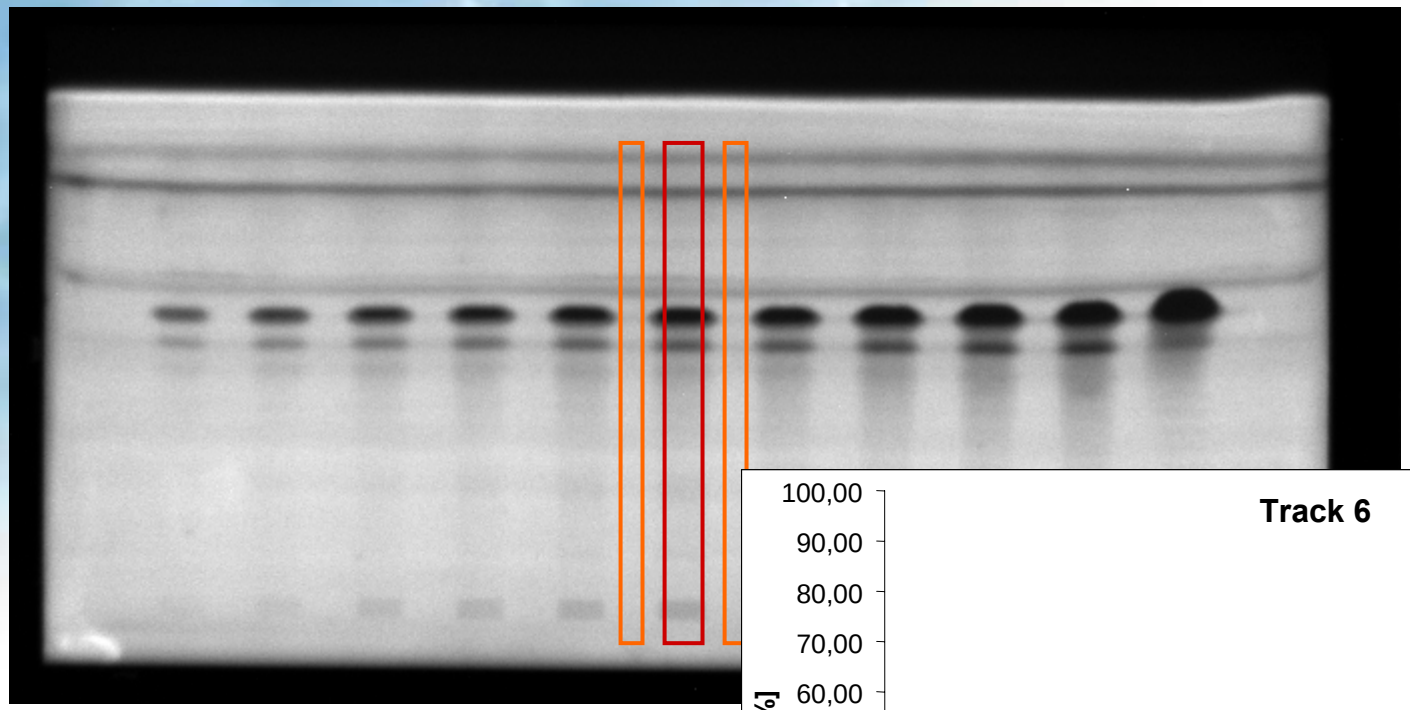

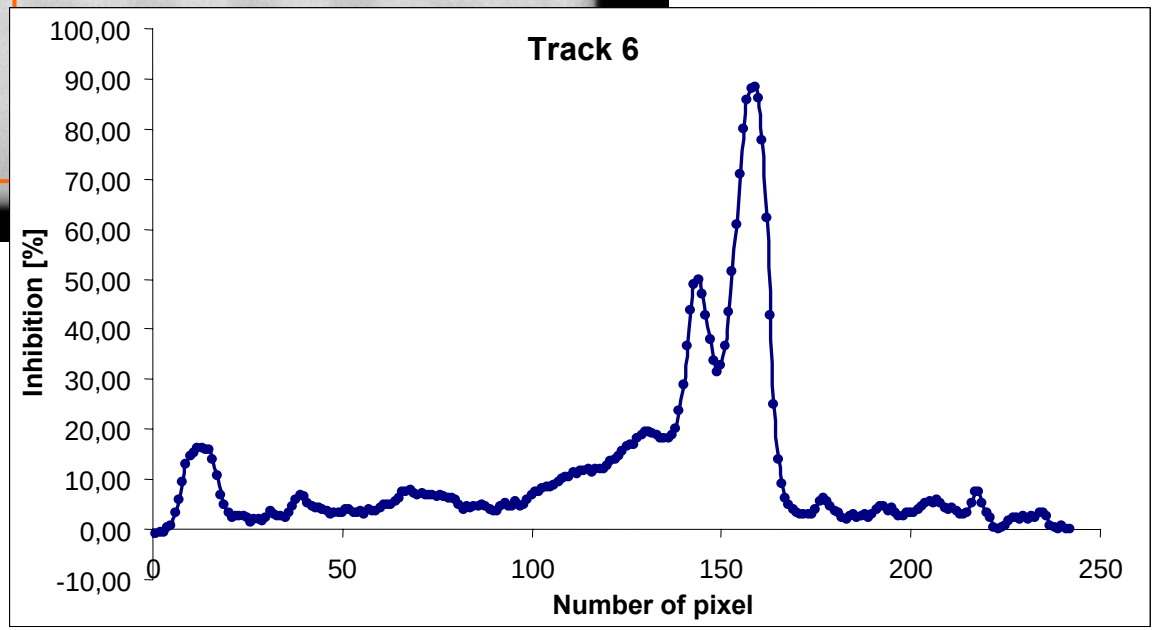

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Comparison of sensitivity of cuvette test and HPTLC test

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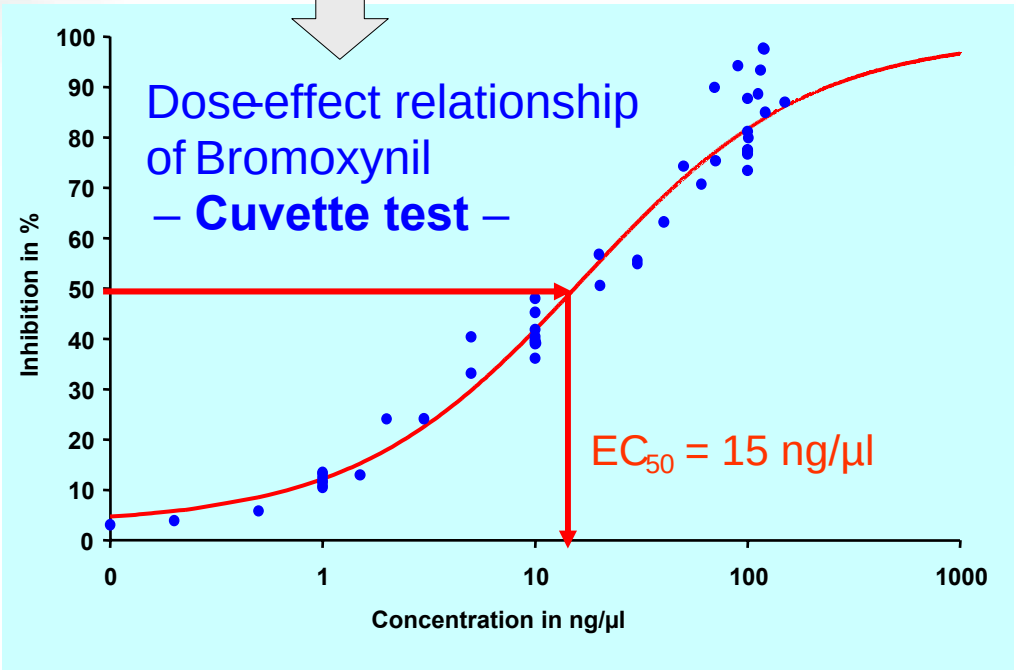
(1) LUMIStox300
(2) LUMIStherm



$$R = c + \frac{1-c}{1+e^{a-b\cdot\ln(Dosis)}}$$

R: Response (inhibition)
c: Control
a: Location parameter
b: Slope parameter

$$EC_{50} = e^{a/b}\cdot(1-2\cdot c)^{1/b}$$

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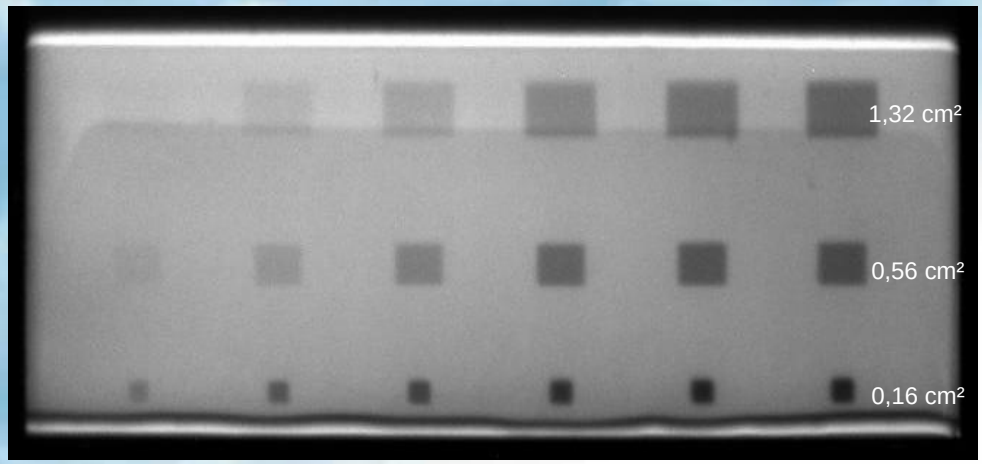
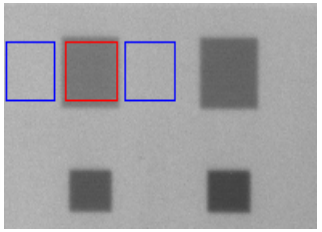
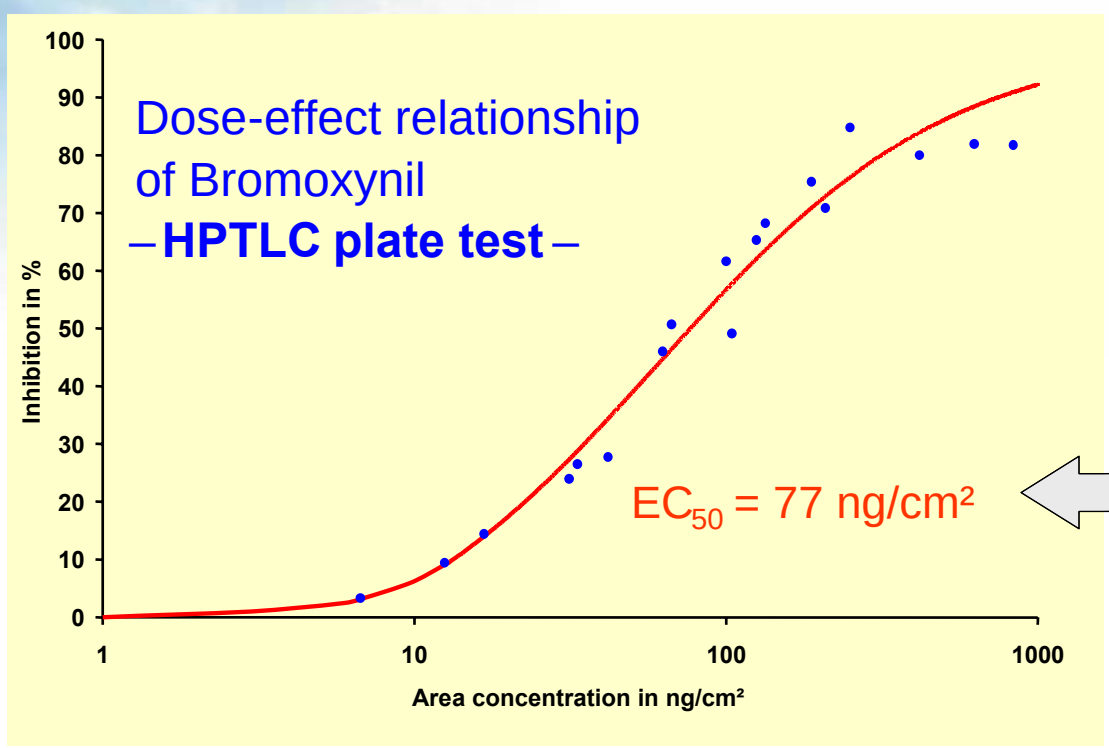
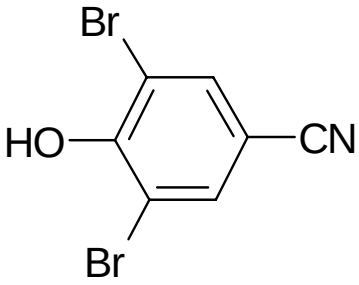


$$I^{S} = 1 - \frac{\sum_{n_1}^{n_2} i_n^{S}}{\sum_{n_1}^{n_2} i_n^{R}}$$

I = Inhibition
S = Sample
R = Reference
i = Intensity
n = Pixel number







Calculation of the iso-inhibition value curve

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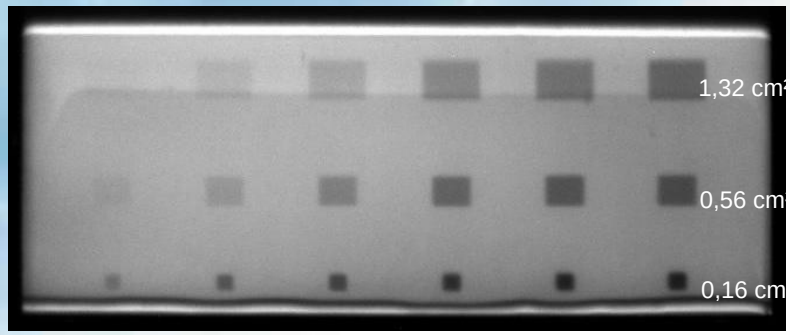
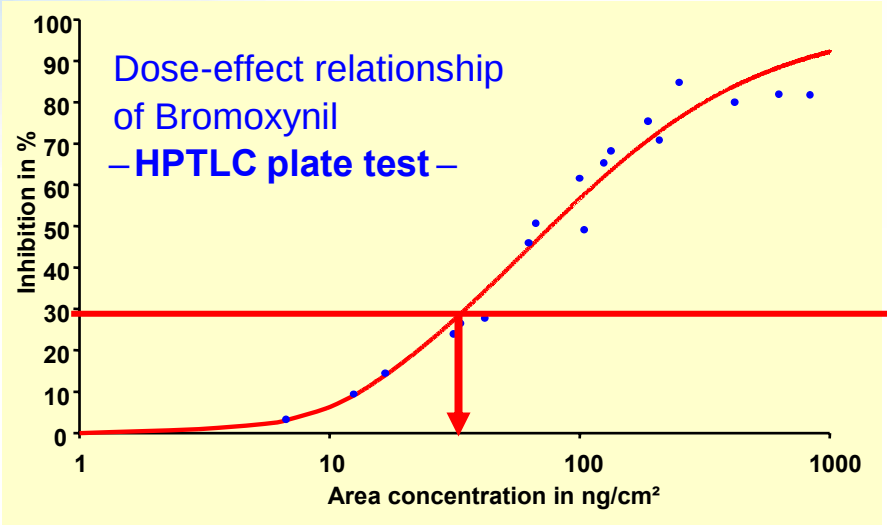
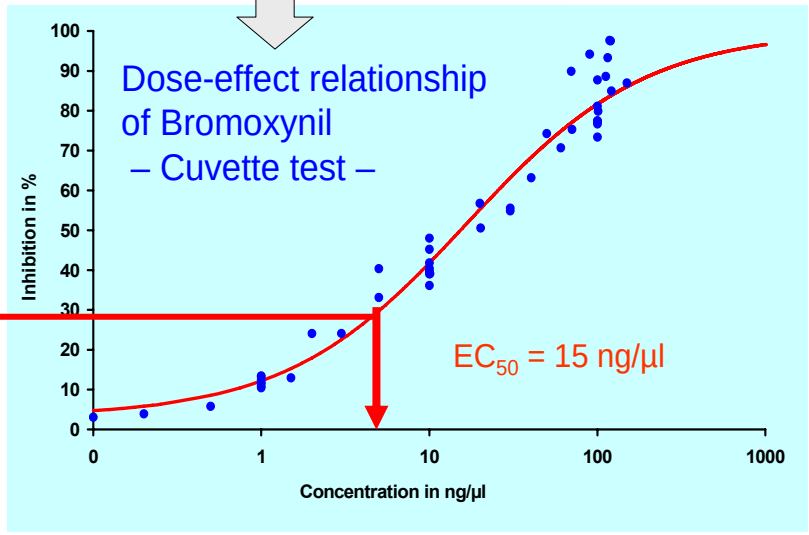


(1) LUMIStox 300
(2) LUMIStherm





Curve of iso-inhibition values

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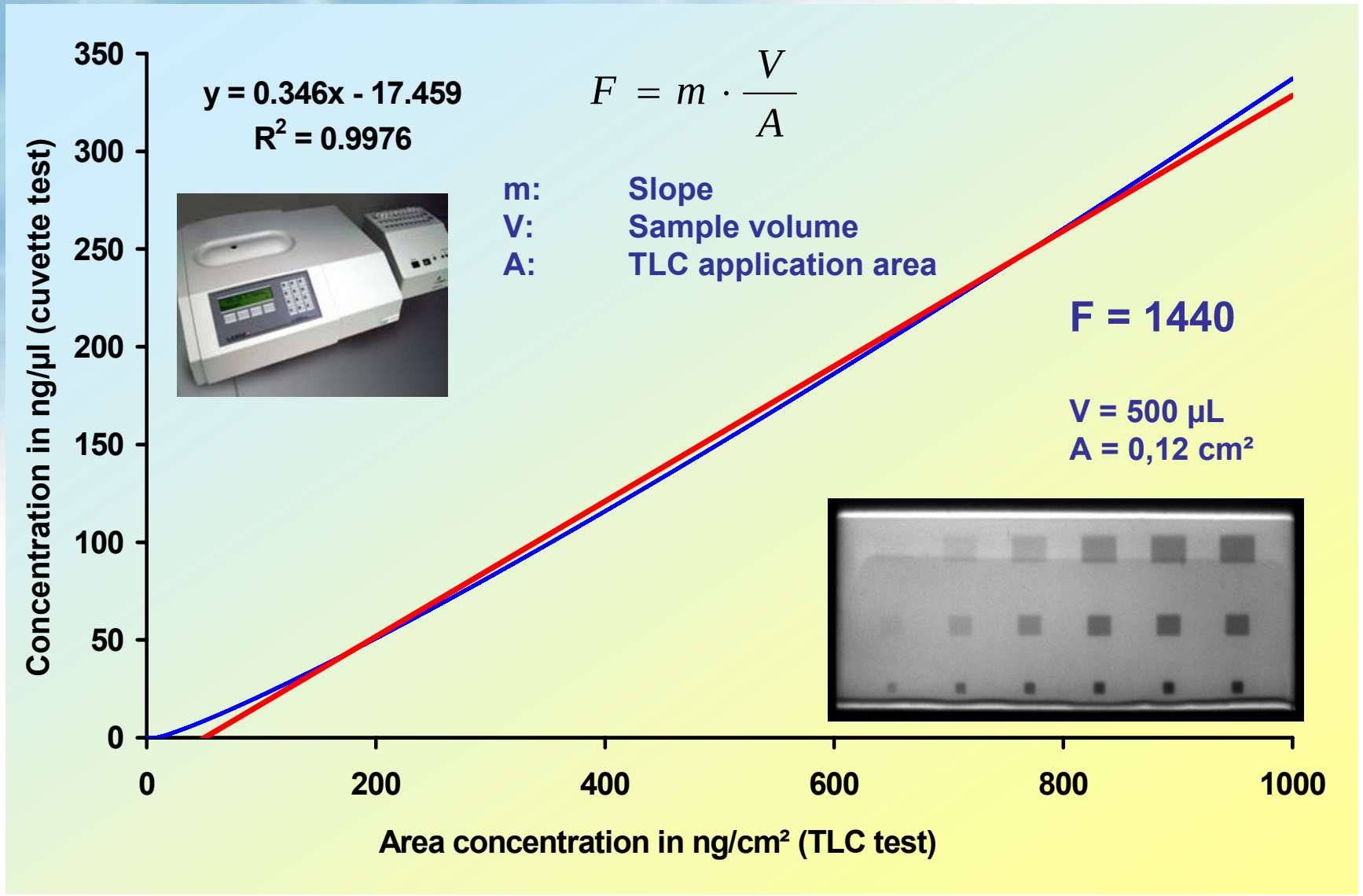

Sensitivity factor for selected substances

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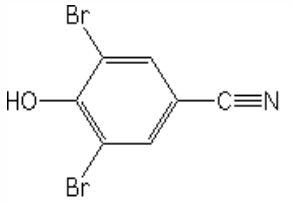
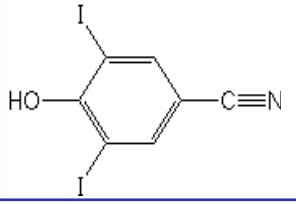
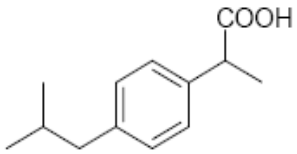
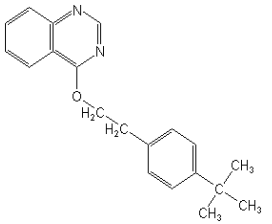
Substance	*Sensitivity factor*	*Structure*
Bromoxynil	**ca. 1440**	
Ioxynil	**ca. 311**	
Ibuprofen	**ca. 180**	
Fenazaquin	**ca. 1650**	

Effect of incubation time on the inhibition of luminescence

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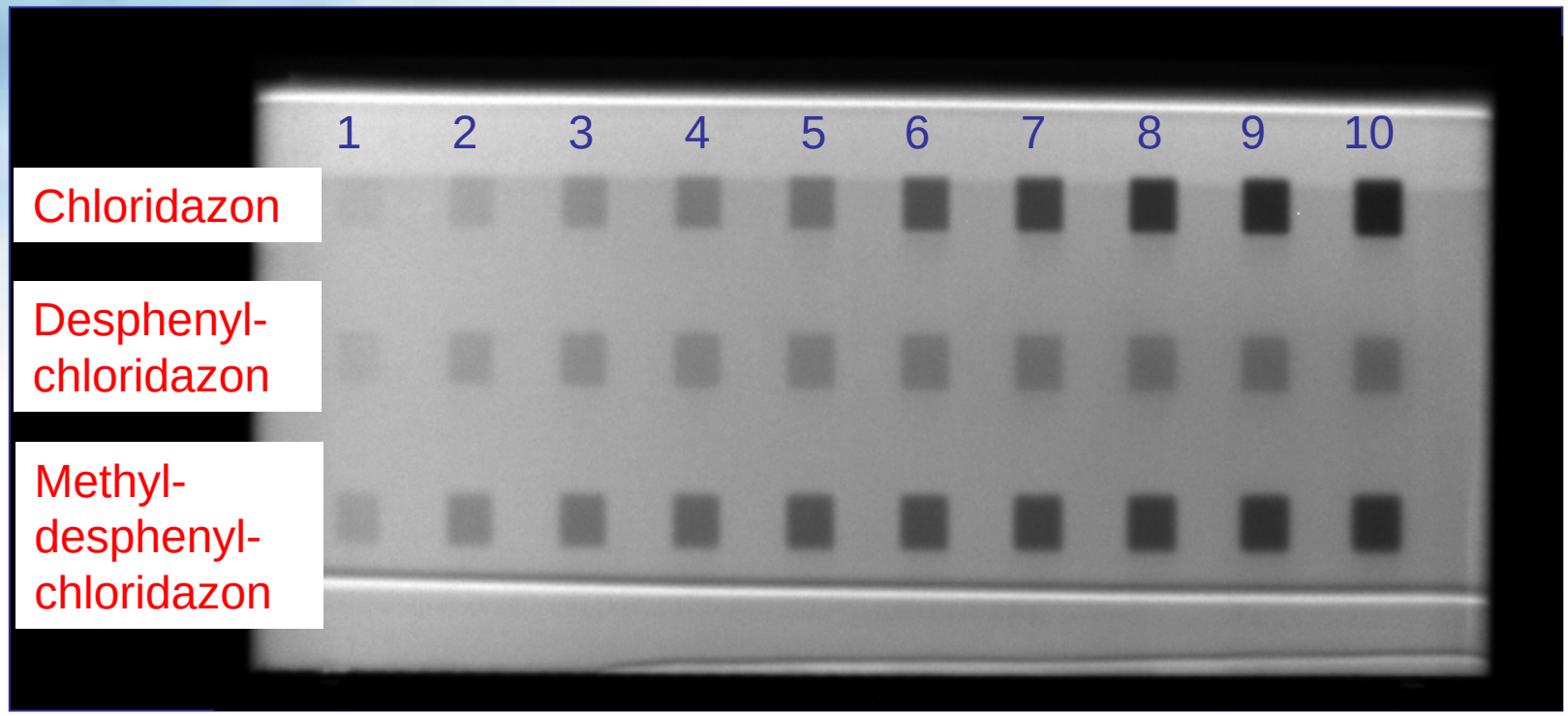

Standard incubation time: 10 min

Chloridazon and metabolites

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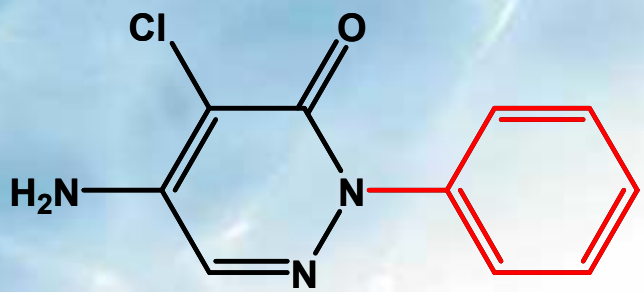
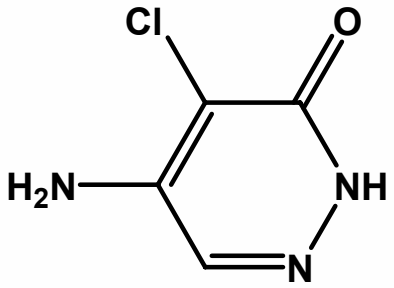


Chloridazon

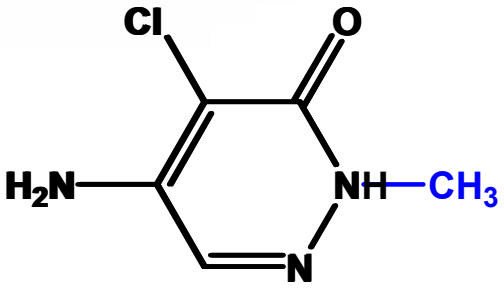
- Herbicide
- Application for sugar beets
- Used since 1964
- In 2005 approved for further 10 years in Germany

Degradation in soil



Desphenyl-chloridazon (Metabolite B)

Transformation in soil



Methyl-desphenyl-chloridazon (Metabolite B1)

Comparison of different incubation times

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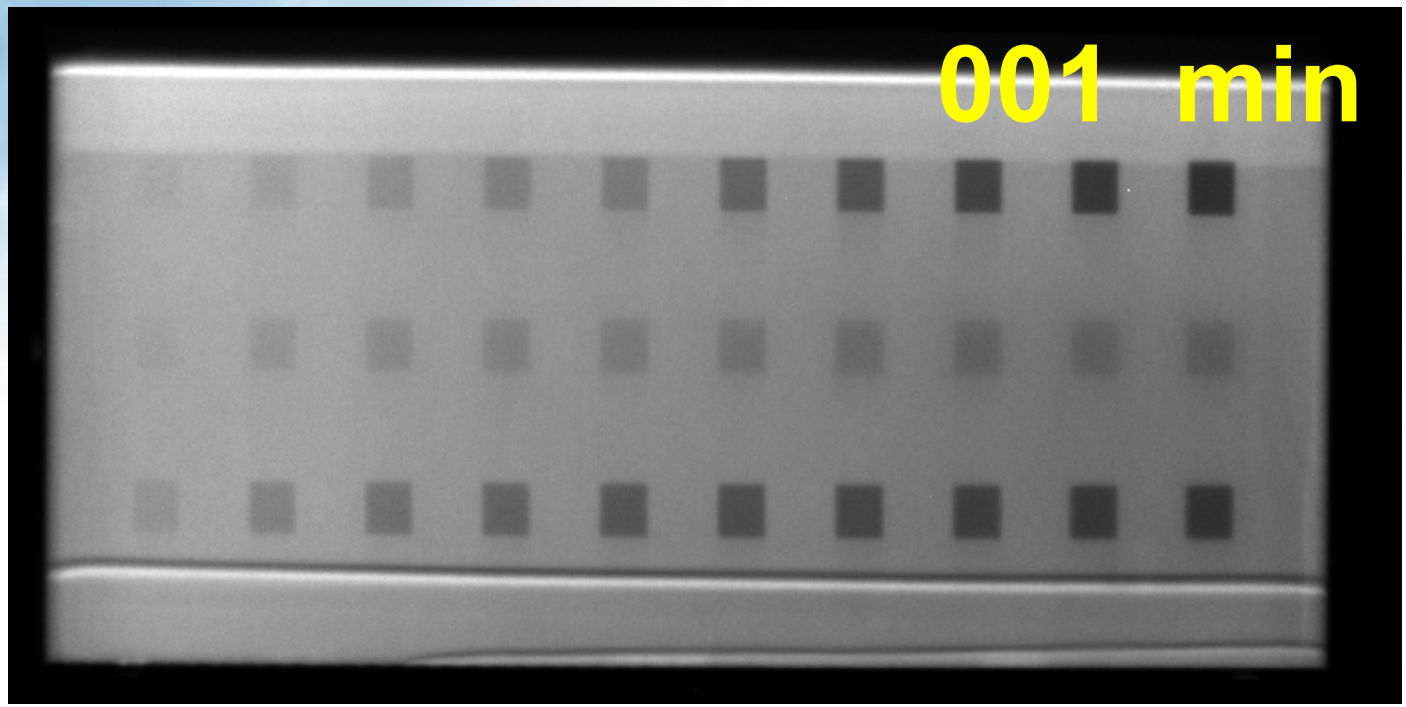

Comparison of different incubation times

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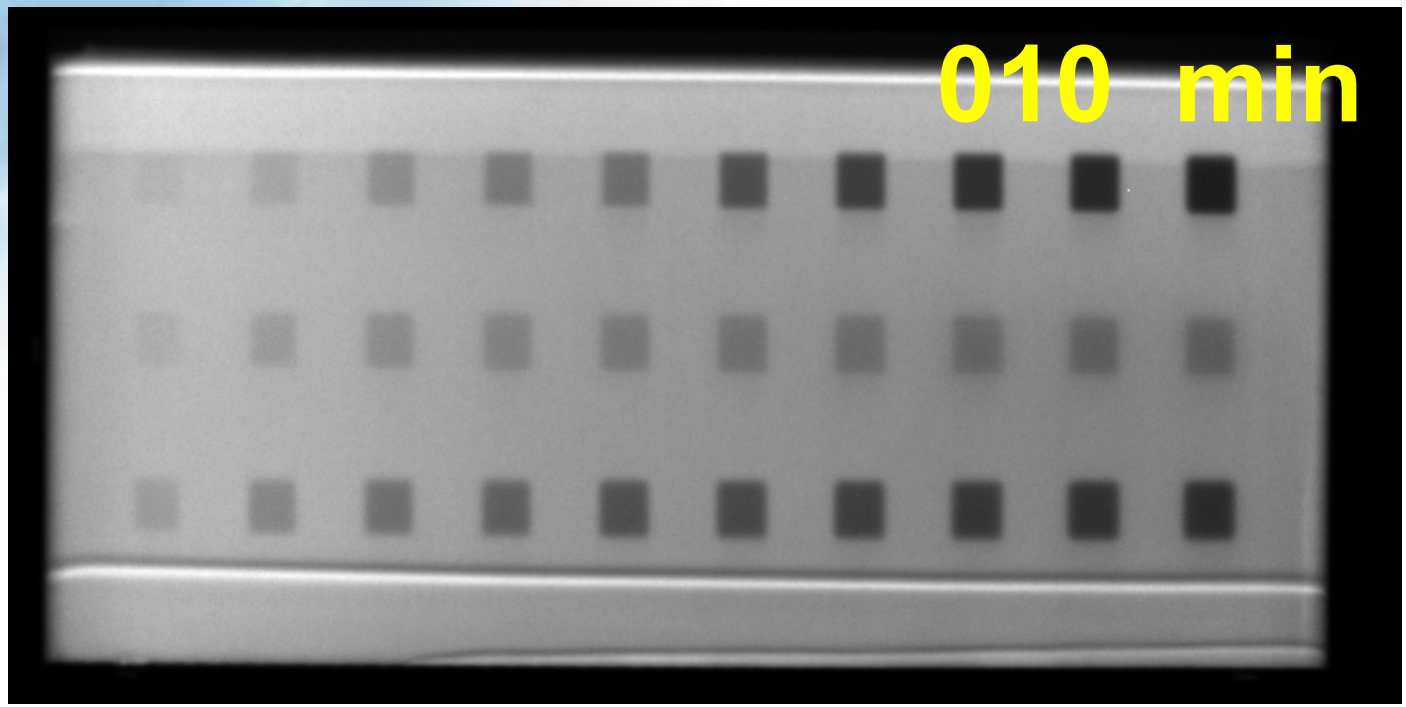

Comparison of different incubation times

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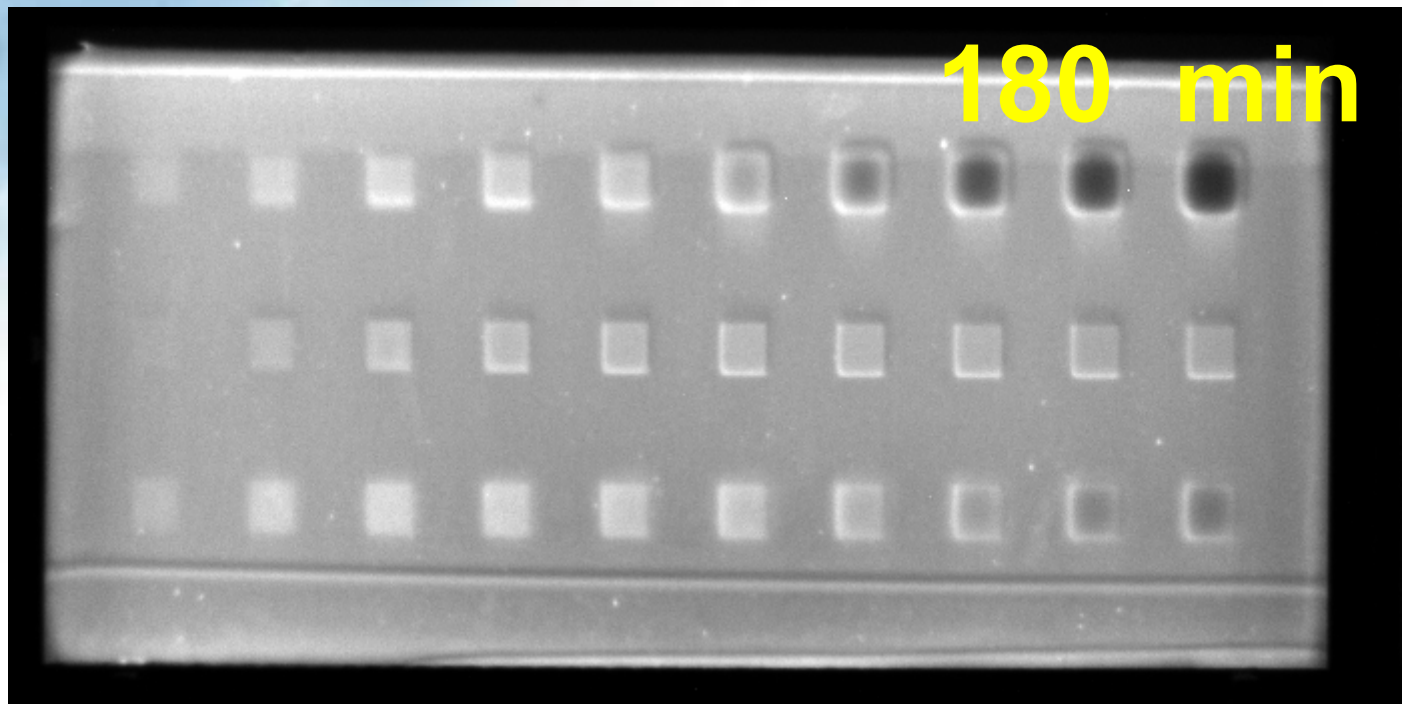

Comparison of different incubation times

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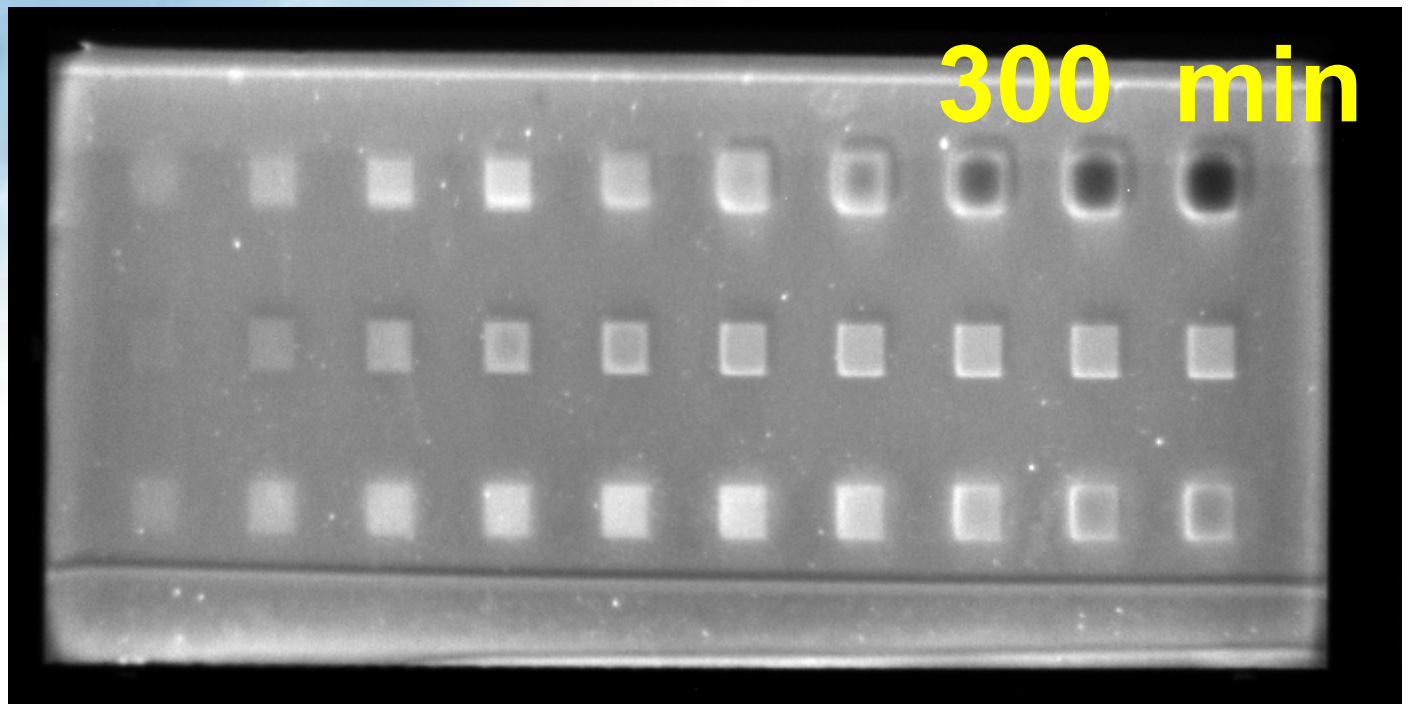

- Luminescence inhibition may change to intensification

Comparison of different incubation times

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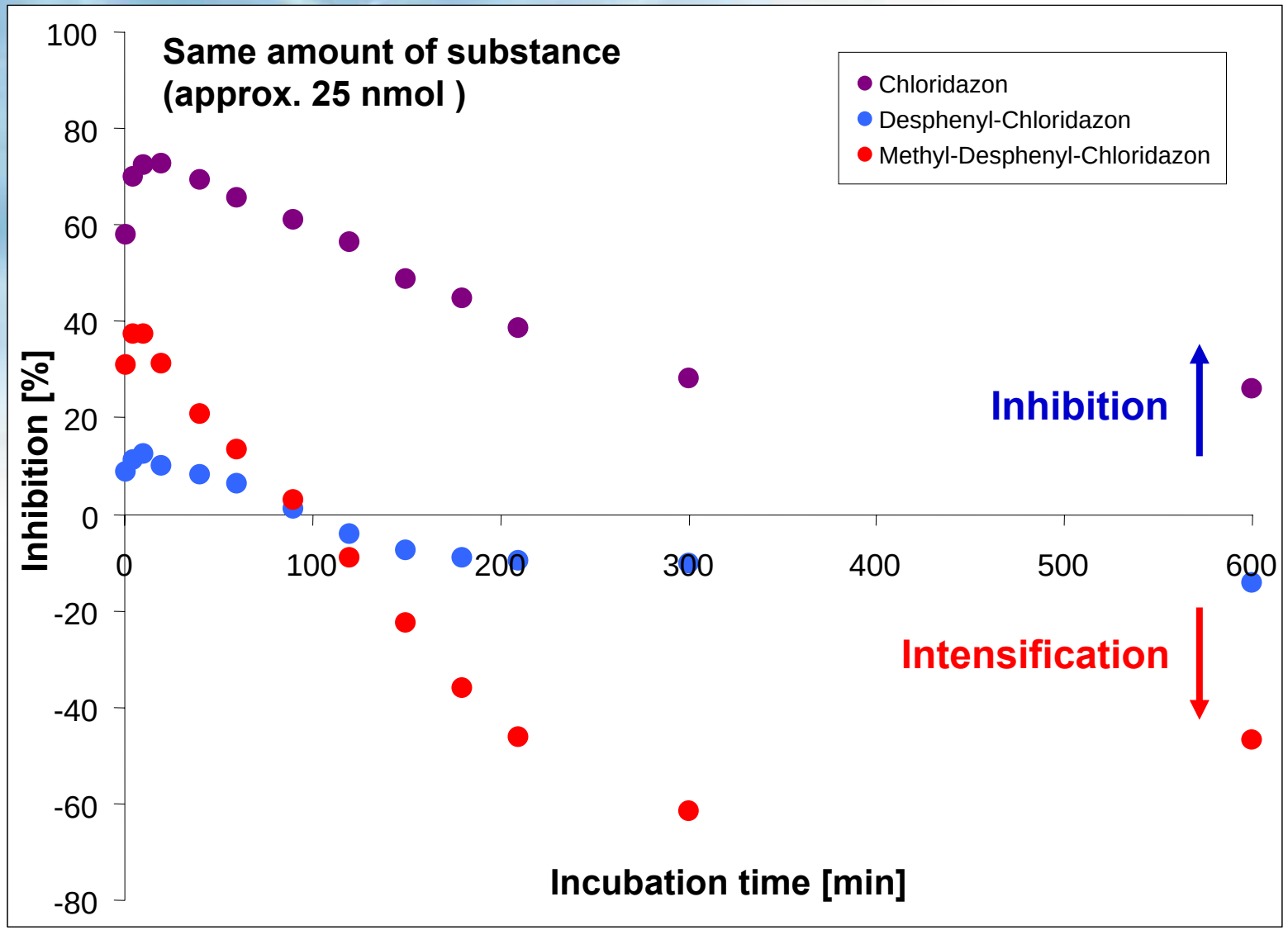

Application example: Potential ground water contamination by coatings of sports fields

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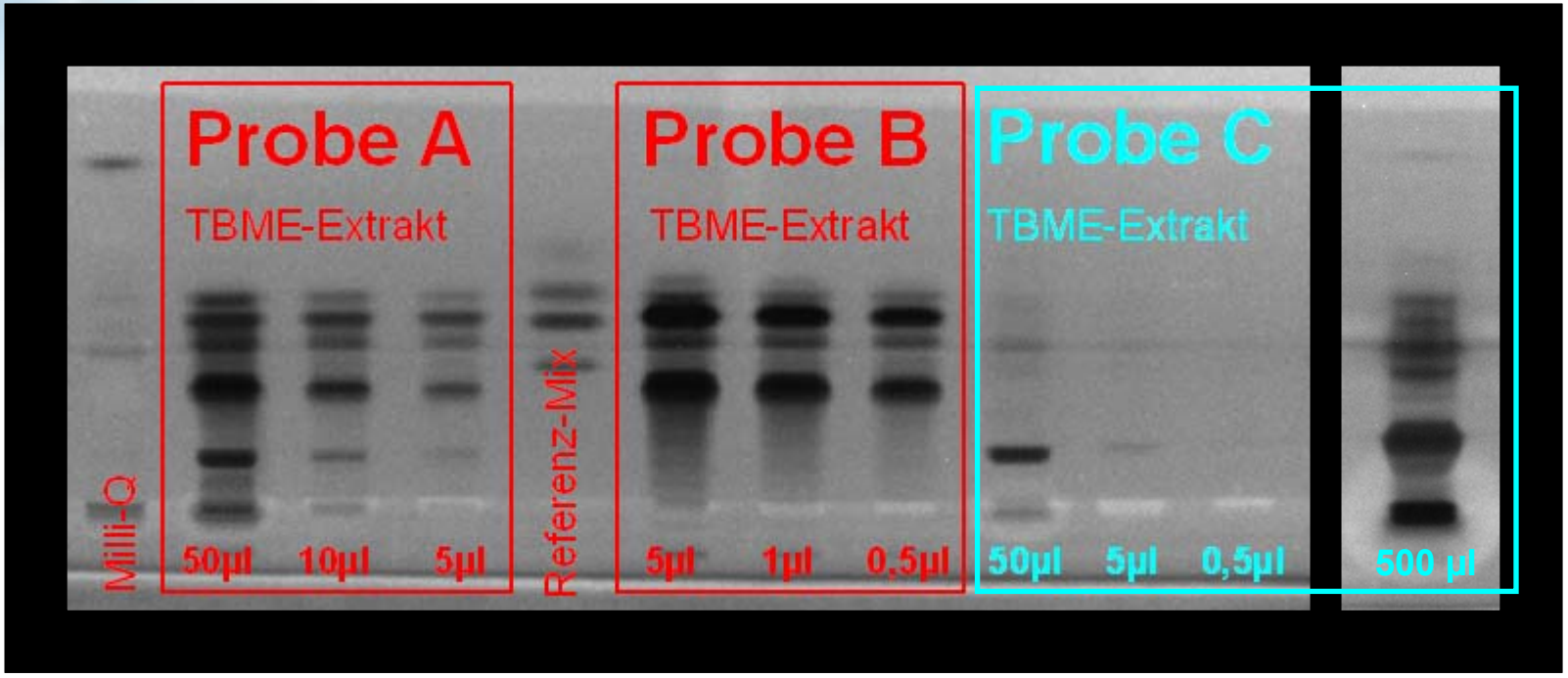
Sample preparation:

- 40 g granulate in 80 g H_2O 24 h shaking
- Eluate filtered (0,4 µm membrane)
- Liquid-liquid extraction with TBME → approx.. 1 ml extract



Application example:
Bioactivity test of ozonation by-products of beta-blocker substances

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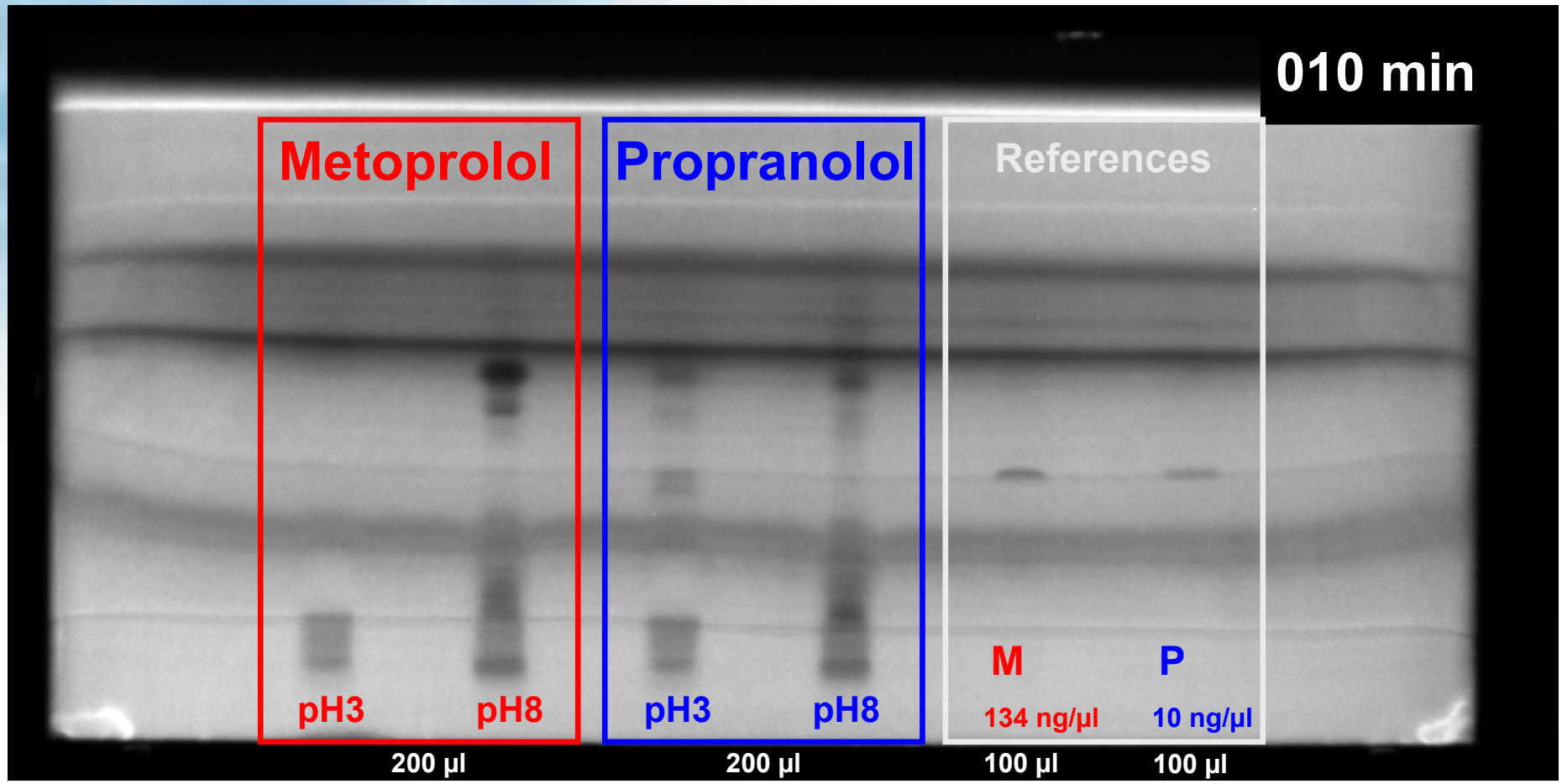

Application example: Fingerprint of waste water samples (Δt = 6 month)

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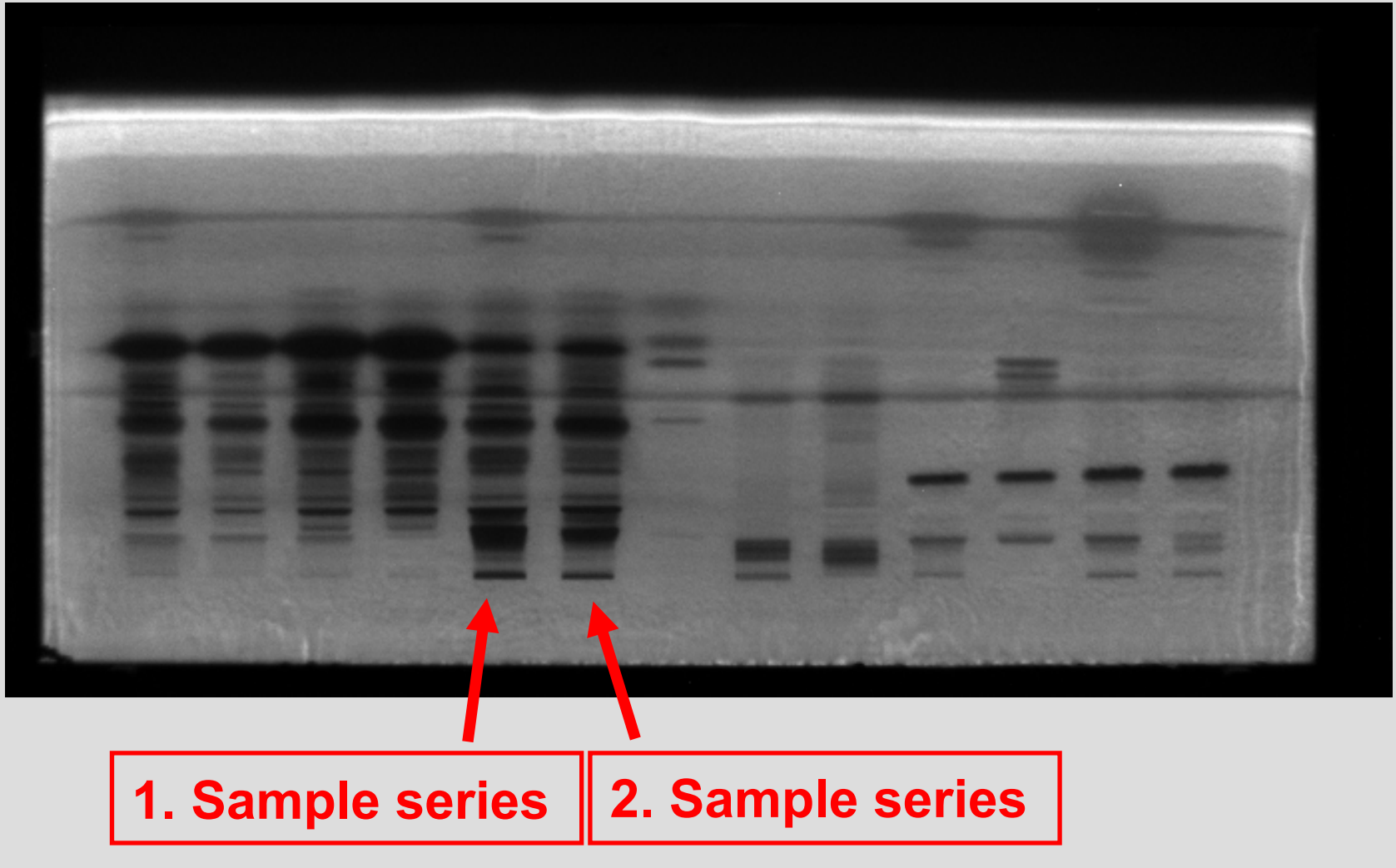

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Advanced data evaluation

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- In general, quantitation requires reference standards
  - ➢ What a about unknown compounds?
- The **semi-quantitative** evaluation of the HPTLC chromatograms with bioactivity detection required a **new data evaluation procedure**:

(1) Linearization of the dose-effect relationship

(2) Calculation of the iso-inhibition volume V(50)

(3) Introduction of the reciprocal iso-inhibition volume 1/V(50)

- ➢ This enables the comparison of the concentration of the same unknown substance in different samples

(1) Linearization of the dose-effect relationship – Gamma value

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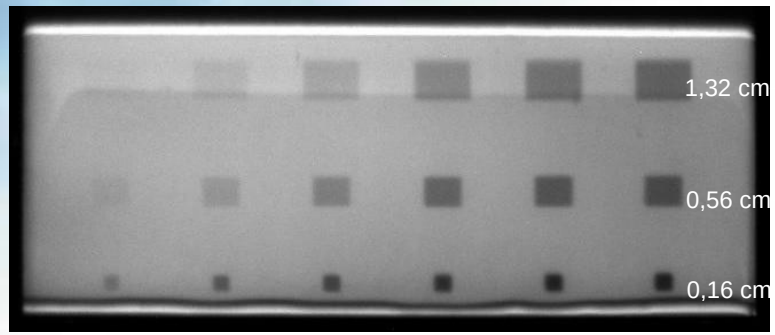
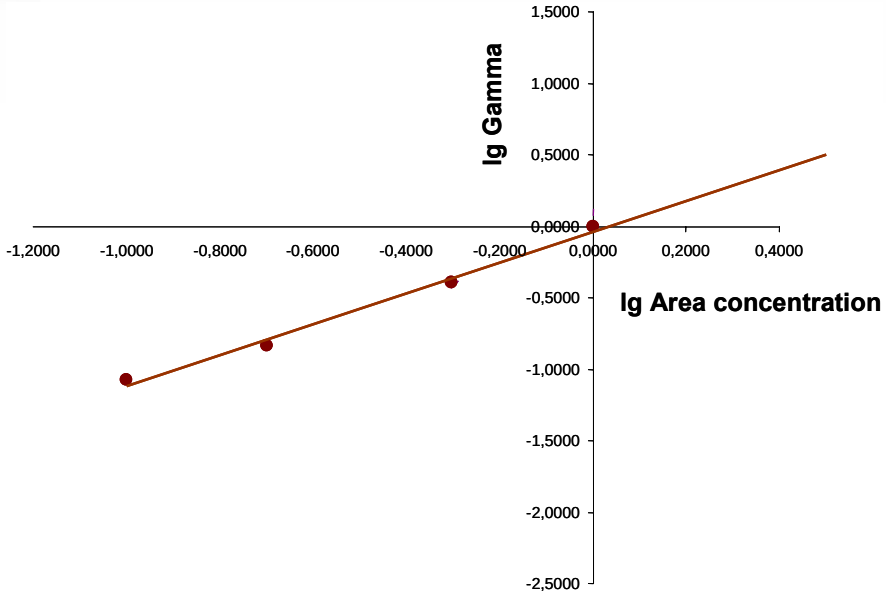
Gamma value $\Gamma = \dfrac{I}{100 - I}$





Linearised dose-effect relationship

$$\lg\Gamma_{i,j} = a_{i,j} + b_{i,j} \cdot \lg V_{i,j}$$

I = *Inhibition (%)*

V = *Application volume (µl)*

$\Gamma_{i,j}$ = *Gamma value*

i = *Substance zone*

j = *Extract (sample)*

(1) Linearised dose-effect relationship

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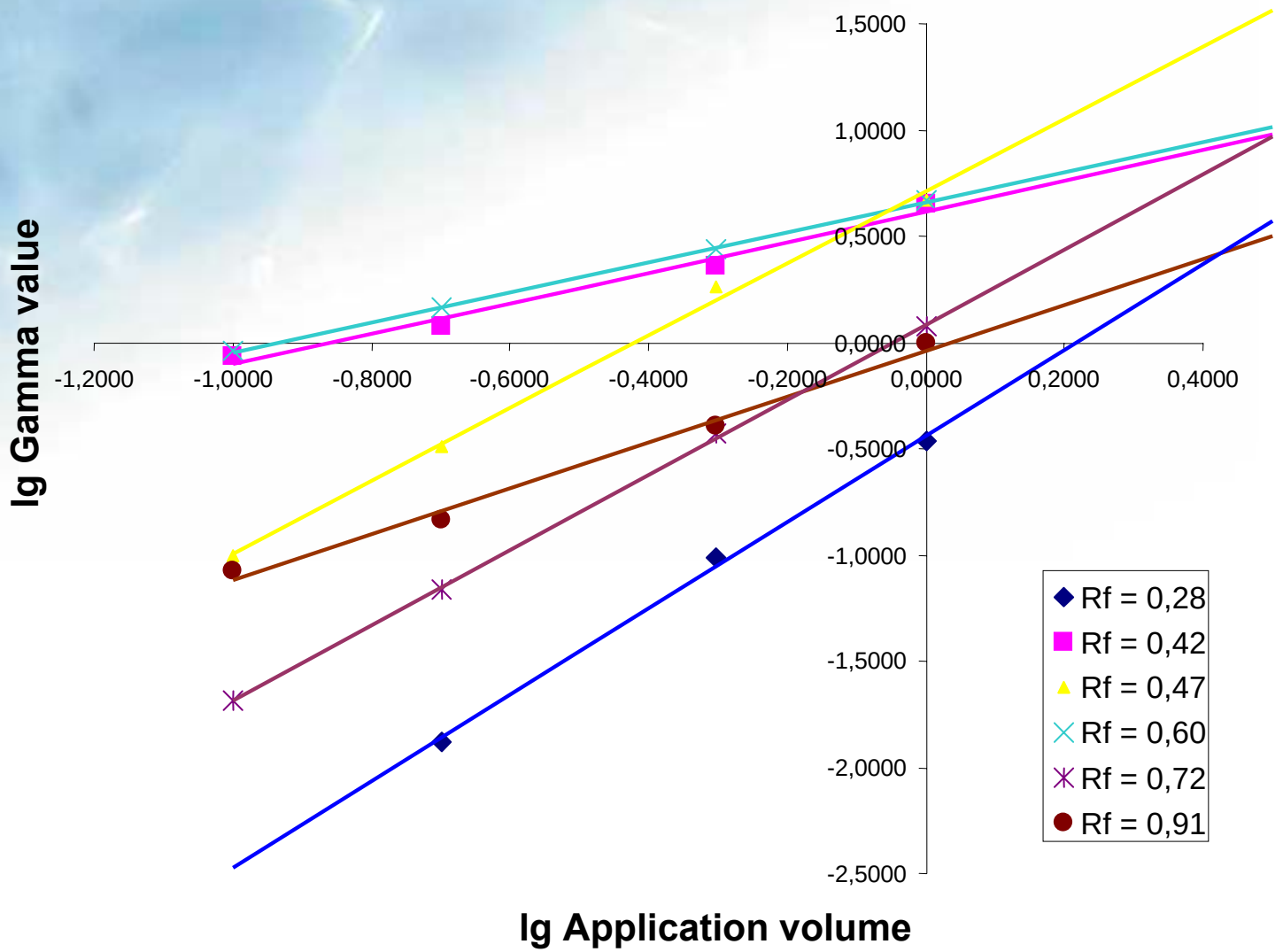

(2) Calculation of the iso-inhibition volume

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Gamma value $\Gamma = \frac{I}{100 - I}$

Linearised dose-effect relationship $\lg\Gamma_{i,j} = a_{i,j} + b_{i,j} \cdot \lg V_{i,j}$

Effect concentration 50

$$I = 50 \longrightarrow \Gamma = 1 \longrightarrow 0 = a_{i,j} + b_{i,j} \cdot \lg V_{i,j}(50)$$

$$\longrightarrow V_{i,j}(50) = 10^{-a_{i,j} / b_{i,j}}$$ Iso-inhibition volume

$$I_i \approx m_i = c_i \cdot V_i$$

Inhibition is proportional to the applicated sample volume

I = *Inhibition (%)*

V = *Application volume (µl)*

$\Gamma_{i,j}$ = *Gamma value*

i = *Substance zone*

j = *Extract (sample)*

(3) Introduction of the reciprocal iso-inhibition volume

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$$m_{i,1}(50) = c_{i,1} \cdot V_{i,1}(50)$$
$$m_{i,2}(50) = c_{i,2} \cdot V_{i,2}(50)$$

$$m_{i,1}(50) = m_{i,2}(50)$$

The same mass for the same substance and the same inhibition

Comparison of substance i in two samples

$$c_{i,1} \cdot V_{i,1}(50) = c_{i,2} \cdot V_{i,2}(50)$$

c = Concentration (ng/µl)

V = Application volume (µl)

i = Substance zone

$$\frac{c_{i,1}}{c_{i,2}} = \frac{\frac{1}{V_{i,1}(50)}}{\frac{1}{V_{i,2}(50)}}$$

Reciprocal iso-inhibition volume

Comparison of different TBME extracts from wastewater

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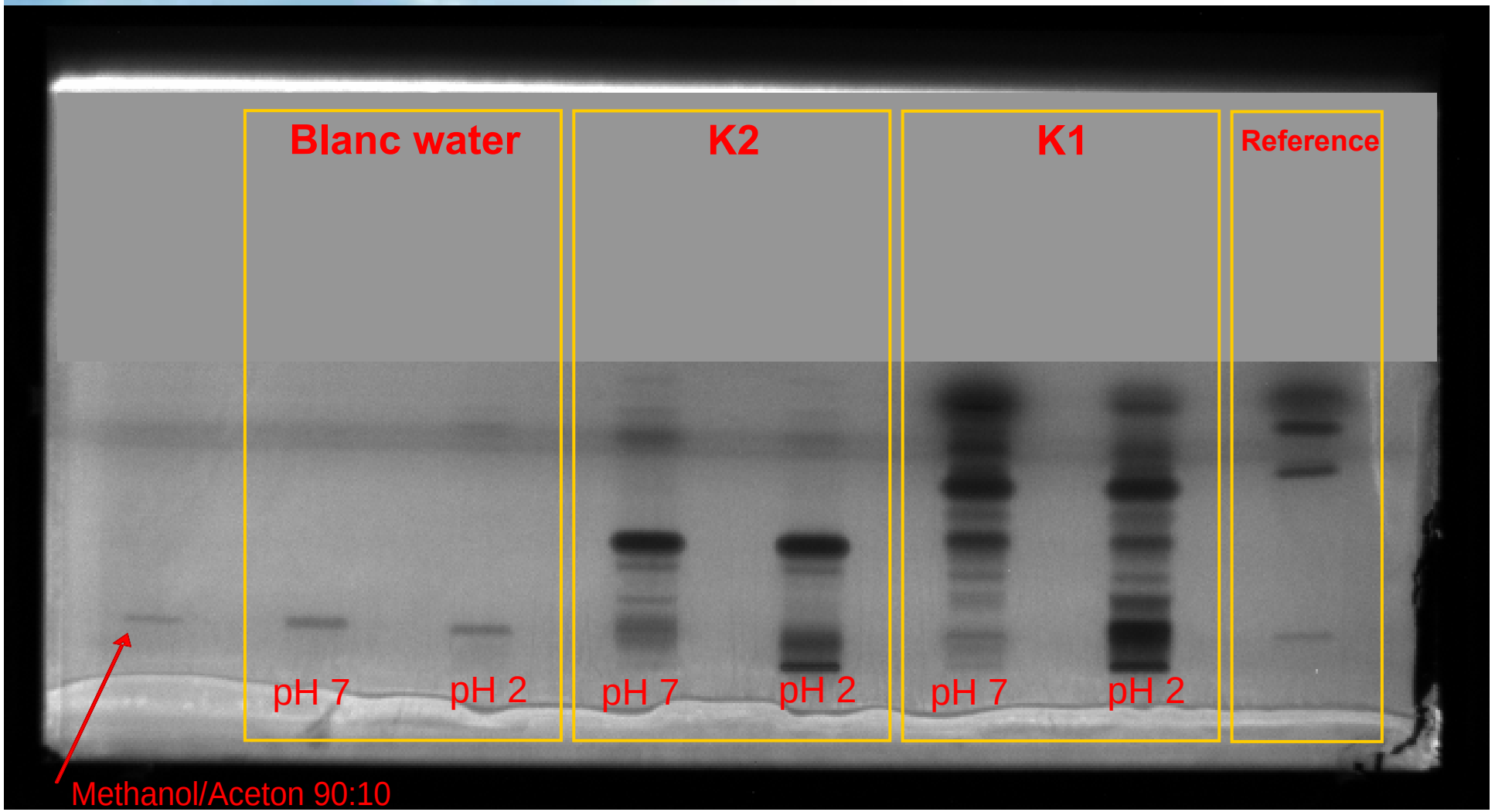

Comparison of two extracts from wastewater using reciprocal iso-inhibition volumes

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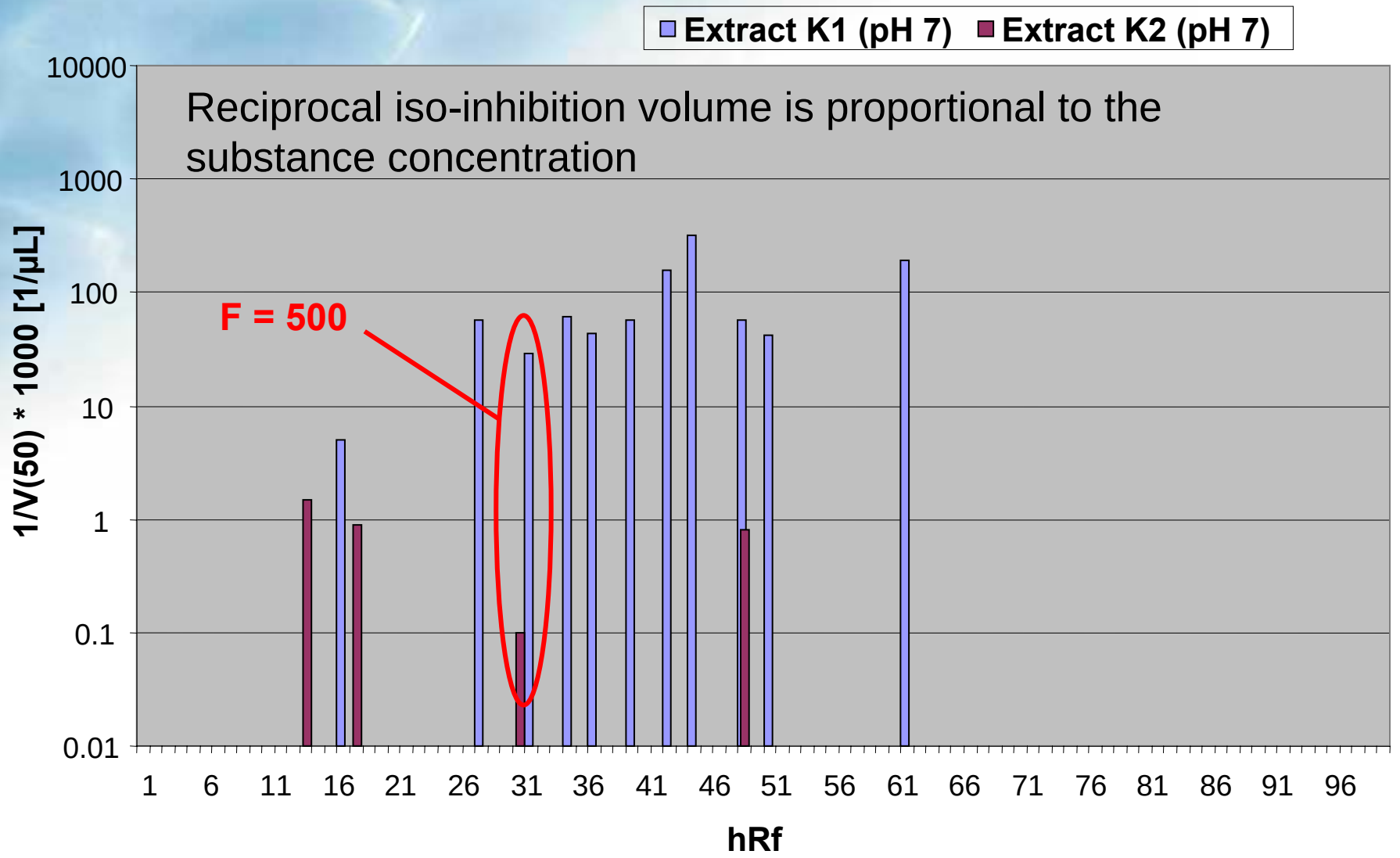

Conclusions

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- Application of luminescent bacteria in HPTLC
  - ➢ Screening for bioactivity of single substances even in complex mixtures possible
- *Vibrio fischeri* HPTLC test shows high sensitivity compared to the classic cuvette test
- Incubation time proved to be an essential parameter
  - ➢ Luminescence inhibition may change to intensification
- New semi-quantitative evaluation procedure applying the concept of "reciprocal iso-inhibition volume"
  - ➢ Comparison of different samples by means of inhibition pattern
  - ➢ Evaluation of the concentration ratios of a substance in different samples

Thank you for your attention!

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