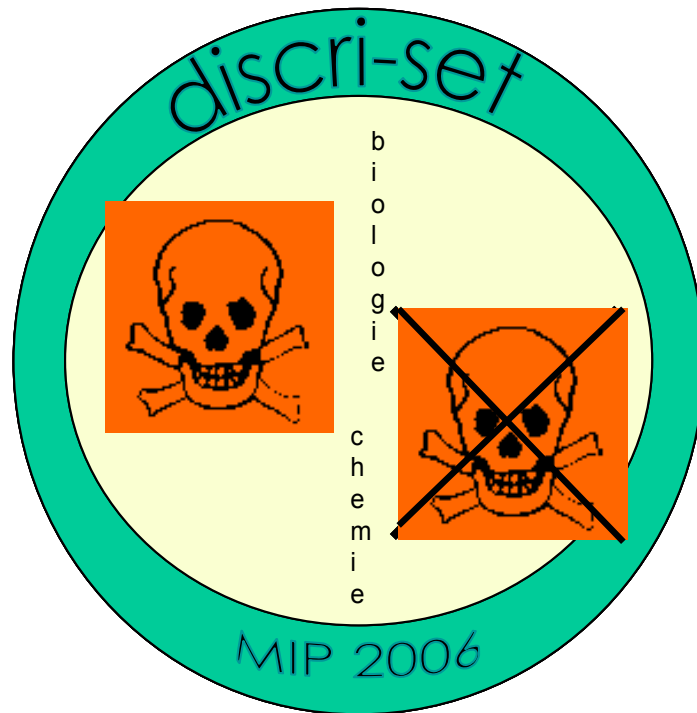


Project: DISCRISET



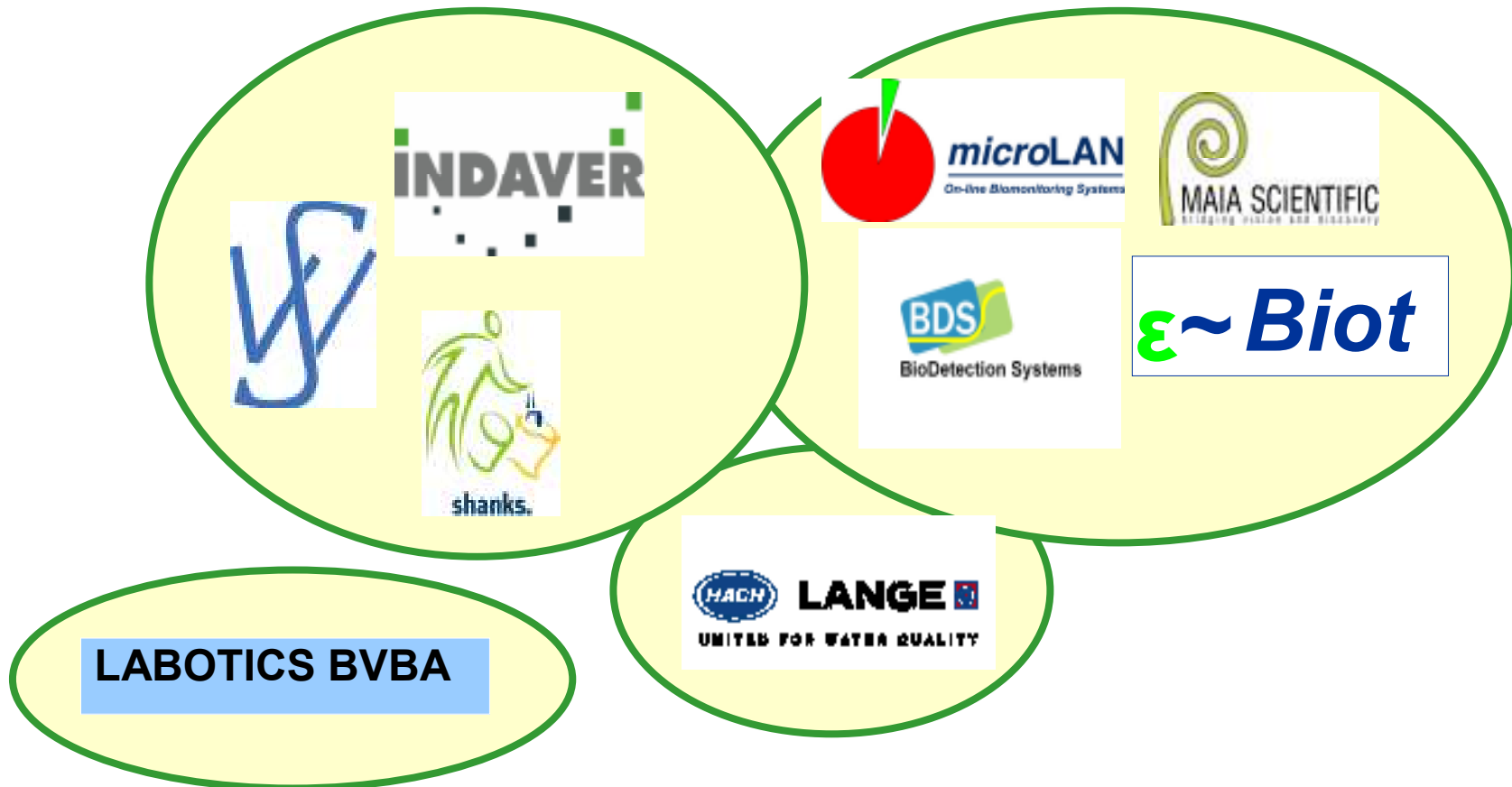
Selecting a Set of (biological/chemical) fast screening tests that allow to discriminate between hazardous and non-hazardous waste!

VITO, UNIVERSITEIT OF HASSELT, UNIVERSITY OF ANTWERP

Supported by: The Environmental Innovation Platform (MIP – Flanders)

Supported by OVAM (waste agency of Flanders)

Industrial partners



Introduction

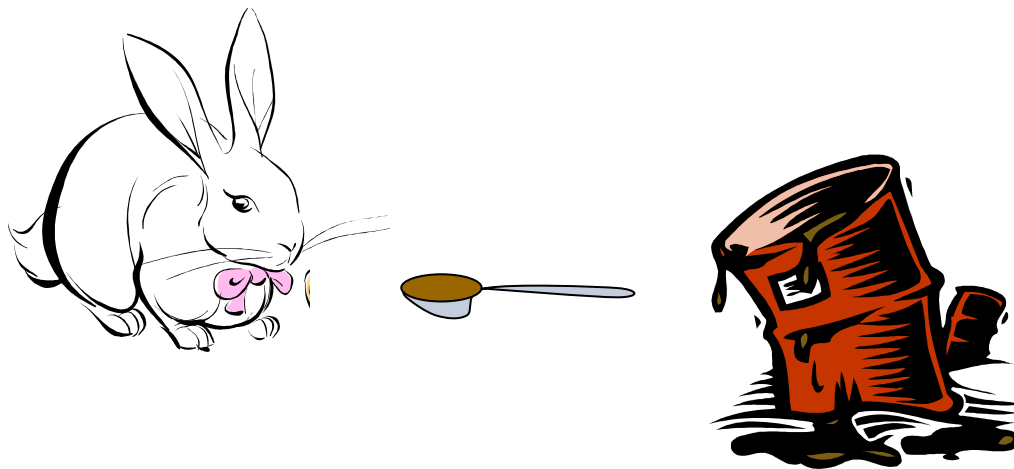
HWD Waste classification:

- Based on type and composition (hazardous properties of individual components)
- Complex wastes: if composition is unknown
 - Test the hazardous properties of the waste (H-criteria) -> **Annex V of Dangerous Substances Directive 67/548/EEG**



Introduction

- **ANNEX V tests are often mammalian tests!!**
these tests cannot be used for hazard characterisation of waste materials (unethical, long test duration, expensive..)



Introduction

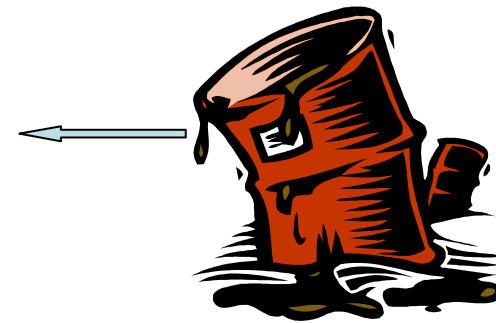
DISCRISET:

- use of *in vitro alternative* methods: faster (aim: max 48 hours), cheaper, no ethical objections

Cell based assays

General toxicity

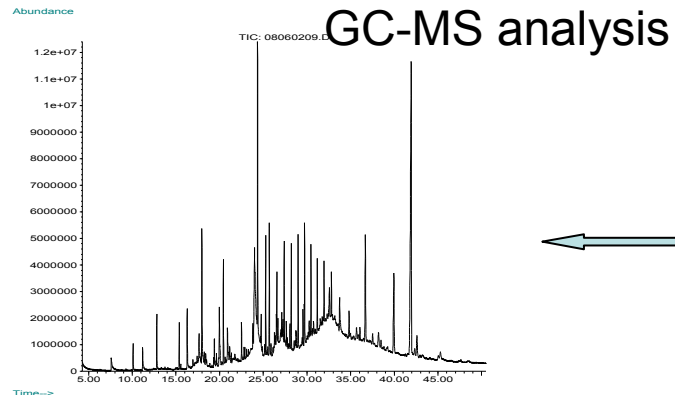
Mechanistic toxicity



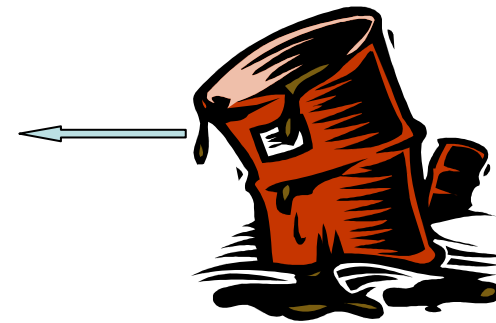
Introduction

- DISCRISET: use of *in vitro alternative* methods: faster (aim: max 48 hours), cheaper, no ethical objections
- + targeted chemical analyses

ED-XRF analysis
Spectrophotometric +
elemental analysis

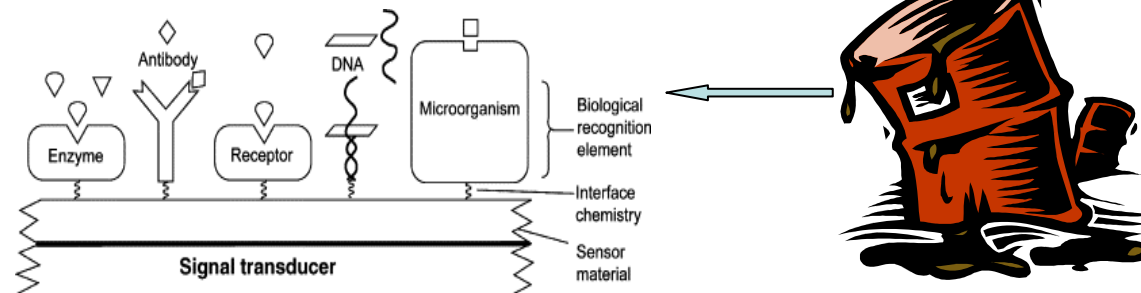


KITS for specific
parameters



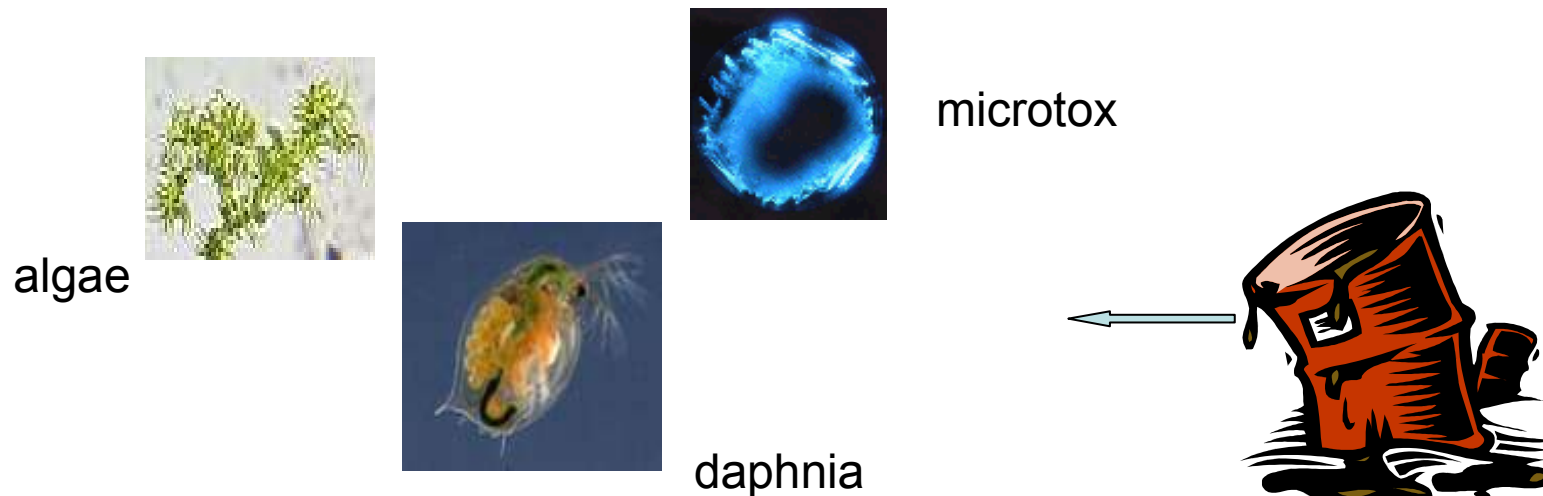
Introduction

- DISCRISET: use of *in vitro alternative* methods: faster (aim: max 48 hours), cheaper, no ethical objections
- + targeted chemical analyses
- + affinity bioassays for targeted analyses



Introduction

- DISCRISET: use of *in vitro alternative* methods: faster (aim: max 48 hours), cheaper, no ethical objections
- + targeted chemical analyses
- + affinity bioassays for targeted analyses
- + ecotoxicity tests



Introduction: Hazardous properties (HWD-limits)

FOCUS Discriset



H	criterion
H1	explosive
H2	oxidising
H3a	highly flammable
H3b	flammable
H4	Irritant
H5	harmful
H6	toxic
H7	carcinogenic
H8	corrosive
H9	Infectious
H10	Toxic for reproduction
H11	mutagenic
H12	release of (very) toxic gases
H13	Leachate with hazardous properties
H14	ecotoxic

Test strategy: step wise



Step 1: Inorganic analyses



Classification based
on inorganic parameters?

yes



Hazardous, no
further testing



no

Step 2: *Relevant* fast screening for main organics using affinity assays/classic methods



Classification based
on these organic parameters?

yes



Hazardous, no
further testing



no

Step 3: Effect assays for mechanisms of toxicity

methods: samples

sample	type
18	filter press textile
19	filter press paint
20	filter press food
21	filter press food
22	filter press food
23	filter press paint
24	filter press paint
27	Shredder PCB
28	Shredder fluff
29	sluge domestic WTP
30	fly ashes
31	wood
32	bottom ashes
33	sludge industrial WTP
75	bottom additive unknown
76	bottom additive unknown

methods



- Sample + water 1/10 -> leachate -> inorganic analyses
- Sample + acetone 1/1 -> organic extract -> substitution by DMSO (10 equivalents /ml) -> organic analyses + biotests

- Biotests:
 - General toxicity: BGPA, microtox, algae, daphnia, cytotoxicity, toxic responses in all test systems
 - Genotoxicity/mutagenicity: Ames, SOS respons (Vitotox, BGPA)
 - Immuno-activity: TNF alpha upregulation
 - Hormonal activity: Calux (see Karolien), YES
 - Teratogenic activity: ELS
 - Ecotoxicity: microtox, algae, daphnia

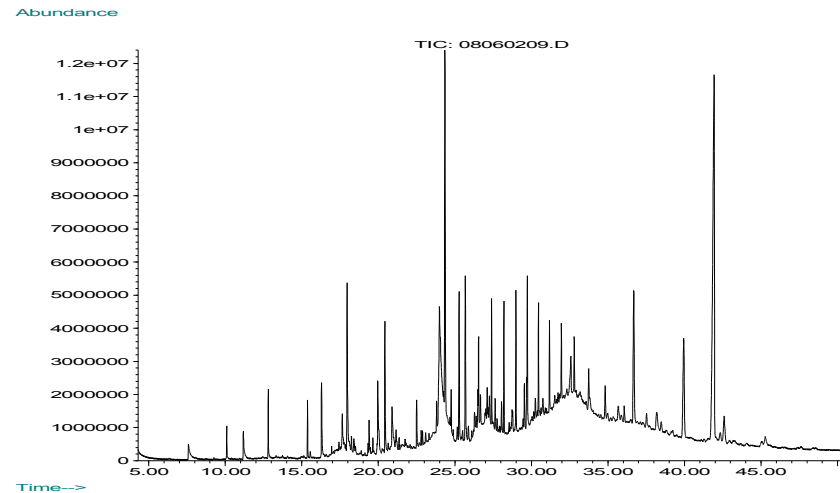
Results: inorganic parameters

sample	type	Al	Si	P	S	Cl	Cr	Co	Ni	Cu	Zn	Br	As
		%	%	%	µg/g	µg/g	µg/g	µg/g	µg/g	µg/g	µg/g	µg/g	µg/g
18	filter press textile	0,34	1,86	0,051	6590	2050	13,2	26,3	24,2	16,4	2130	11,8	nm
19	filter press paint	3,71	2,55	0,85	12370	21100	906	146	692	1370	5600	13960	nm
20	filter press food	0,12	0,3	1,43	9780	6580	29,5	<8	74,2	194	539	248	nm
21	filter press food	0,27	1,38	1,24	6520	708	38,5	15,5	31,6	35,1	233	91,2	nm
22	filter press food	<0,1	0,53	2,2	8500	1730	37,9	29	52,4	66,5	259	38,5	nm
23	filter press paint	1,84	3,49	0,93	4840	6370	181	90,8	765	890	6410	4200	nm
24	filter press paint	3,44	0,76	0,067	7750	9810	746	58	794	327	4120	67480	nm
27	Shredder PCB	2,49	6,82	0,23	12220	15190	465	86	574	7700	20820	nm	64,4
28	Shredder fluff	0,84	4,98	0,239	27370	10890	414	68,8	554	1260	15060	nm	72,3
29	sluge domestic WTP	1,85	20,5	0,17	4460	8140	142	<9,6	18,7	77,9	267	nm	5,7
30	fly ashes	4,83	19,6	0,39	7850	6320	408	89,9	144	2130	3190	nm	15,3
31	wood	<0,05	0,09	100	140	190	200	nm	nm	2020	36	nm	
32	bottom ashes	3,14	21,4	0,497	7190	4330	304	154	423	1630	2900	nm	33,5
33	sludge industrial WTP	2,84	6,9	4,93	8570	880	127	47,7	50	609	1960	nm	17,8

- Not a good tool for classification of hazardous waste
 - Elemental analyses -> limited toxicological information
 - Worst case: assume the element is incorporated in its most toxic molecular structure

Results: organic parameters

- GC-MS -> many peaks;
only a small part can be identified
- Not a good tool for classification of hazardous waste
 - Limited identification -> limited toxicological information
 - Combined toxicity = unpredictable



Results: bioassays

Bacterial tests are the most sensitive, and faster

Microtox is a good candidate as a first step discriminator

sample	type	μtox EC50 (geq/l)	Vitotox EC50(4h) (geq/l)	Daphnia EC50 (geq/l)	Algae % inhibition at 0.1%	ELS EC50 (geq/L)	Cytotoxicity (skin cells) (EC50 72 h)(geq/l)	ames toxicity
19	filter press paint	0,98	0,38	10,36	77,9	>	>	>
19	filter press paint	0,19	0,1	0,05143	97,5	0,1863	0,2	T
16	food	16	-	>	>	>	>	>
	food	1,4	0,25	6,602	76,3	>	>	>
	food	2,02	1,65	8,18	61,3	>	>	>
23	filter press paint	0,14	0,06	0,3106	91	0,1363	0.46	T
24	filter press paint	0,079	0,06	0,2071	85,3	0,1767	0.6	T
27	Shredder PCB	27	nm	>	>	>	>	>
28	Shredder fluff	1,98	nm	2,14	92,6	+++	>	>
29	sludge domestic WTP	5,12	nm	>	>	>	>	>
30	fly ashes	82	nm	>	>	>	>	>
31	wood	0,6	nm	1,65	96	+++	>	T
32	bottom ashes	34	nm	>	>	>	>	>
33	sludge industrial WTP	1,1	nm	3,93	53	+++	>	>
75	bottom additive unknown	>	nm	>	>	>	>	>
76	bottom additive unknown	>	nm	>	>	>	>	>

BGPA general toxicity/genotoxicity

Sample	Oxidative stress	Membrane damage	Cellular stress	DNA Damage
1	20.00	50.00	91.67	26.67
2	0.00	0.00	0.00	14.29
3	2.86	0.00	0.00	0.00
4	0.00	0.00	35.71	0.00
5	5.71	21.43	35.71	8.57
6	14.29	7.14	14.29	5.71
7	20.00	14.29	14.29	31.43

% activated genes

Results: bioassays



b) all end points

EC50 (geq/L)	inorganic	Microtox	Immuno-activity	Hormonal activity	Morphology Fish larvae	Genotoxicity/ mutagenicity	ecotoxicity
H	H5-H6	H5-H6	H4-H8	H10	H10	H7 + H11	H14
1	no	yes	yes	yes	no	yes	yes
2	yes	yes	yes	yes	yes	yes	yes
3	no	no	no	no	no	no	no
4	no	yes	yes	no	yes	yes	yes
5	no	yes	yes	no	no	yes	yes
6	yes	yes	yes	yes	yes	yes	yes
7	no	yes	yes	yes	yes	yes	yes

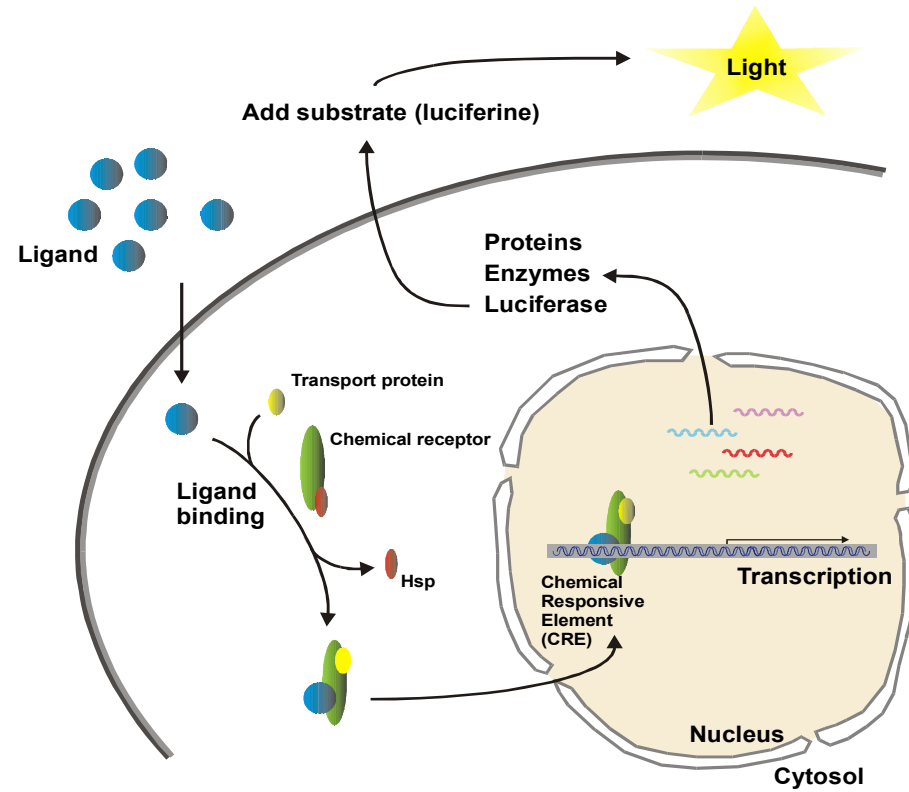
DISCRISET: CALUX ASSAYS

- Phase 1: Calux selected as a part of a battery of biotests for waste assessment
- Phase 2: pilot study on 7 filter cake samples
- Phase 3: broader spectrum of wastes investigated

Chemically Activated LUciferase eXpression



- ER
- ER α
- AR
- DR (AhR)



Experimental set-up (phase 2)

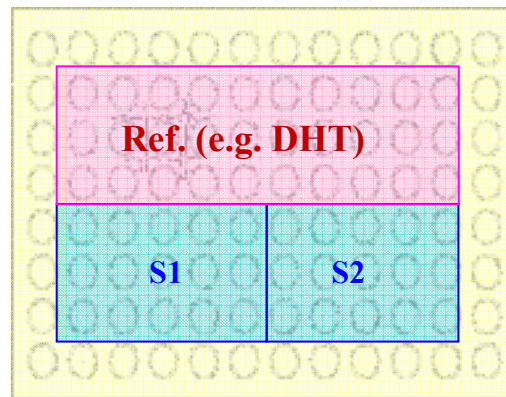


DMSO extracts of waste samples

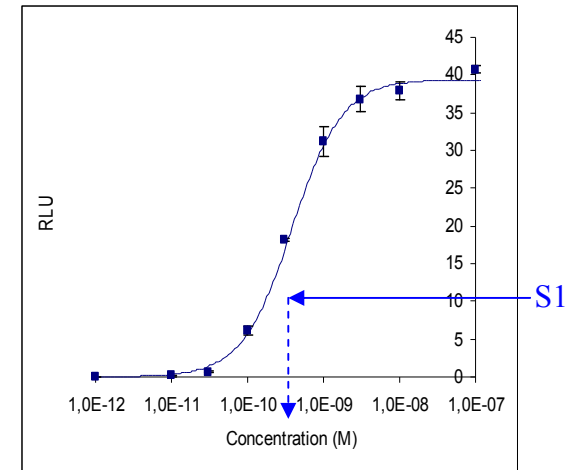
⇒ dilution series:

1 – 100 – 10.000 – 1.000.000 – 100.000.000

⇒ solutions added to cell medium (e.g. 0,1 % for AR Calux)



Incubation Calux cells
24 – 48 h...



Phase 2 results



	DR CALUX 24 hr	DR CALUX 48 hr	AR CALUX anta	ERα CALUX ago	ER CALUX ago	ER CALUX anta
sample	ng TEQ/geq	ng TEQ/geq	ug flutamide/geq	ng E2/geq	ng E2/geq	ug tamoxifen/geq
08E025	1,07	0,403	17	0,12	0,04	7
08E027	4,59	2,119	< 2	< 0,08	< 0,04	1885
08E029	0,01	0,004	< 2	< 0,08	< 0,04	< 1
08E031	0,102	0,033	< 2	< 0,08	< 0,04	< 1
08E033	0,090	0,025	< 2	< 0,08	0,07	< 1
08E035	7,64	3,812	748	4,21	16,44	< 1
08E037	73,68	34,323	2429	4,73	9,54	< 1

Highly sensitive assay

Duration of test: 24 tot 48 hours

Pilot test conclusions



A combination of biotests can provide a tool for hazard assessment of complex waste materials.

Interpretation is needed! => wider range of wastes need to be investigated

= Phase 3

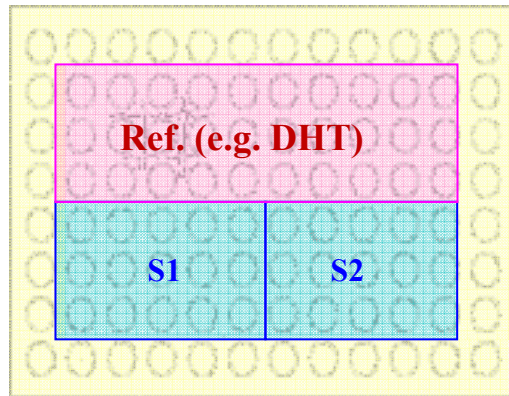
Experimental set-up (phase 3)

different

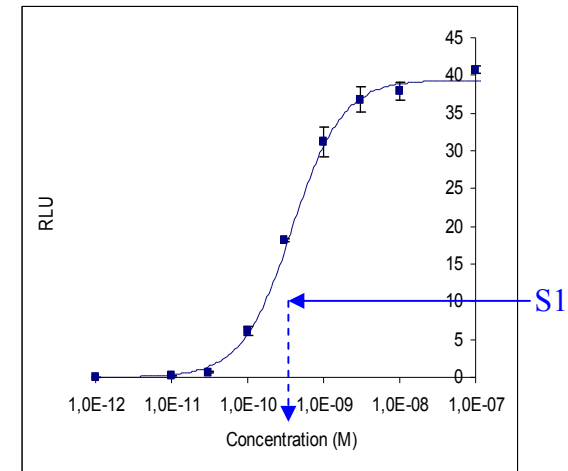


- DMSO extracts of waste samples
- ⇒ dilution series:
- 1 – 100 – 10.000 – 1.000.000 – 100.000.000
- ⇒ This solution was added to cellmedium (e.g. 0,1 % for AR Calux)

+ centrifugated samples



Incubation Calux cells
24 – 48 h...



Finetune dilution series + controls

Phase 3 preliminary results



	DR ng TCDD Eq/geq		ER ng EEQ/geq		AR ng DHT Eq/geq		AR-anta µg FlutEq/geq	
	Not centr.	Centr.	Not centr.	Centr.	Not centr.	Centr.	Not centr.	Centr.
Schredder PCB	53,07	51,33	<LOQ	<LOQ	< LOD	< LOD	105	18
Shredder fluff	70,79	64,41	0,05	<LOQ	< LOD	< LOD	223	< LOD
sluge domestic WTP	2,58	<LOQ	<LOQ	<LOQ	< LOD	< LOD	12	230
fly ashes	0,74	<LOQ	<LOQ	<LOQ	< LOD	< LOD	< LOD	< LOD
wood	0,41	0,28	3,75	<LOQ	< LOD	< LOD	340	< LOD
bottom ashes	0,90	0,91	<LOQ	<LOQ	< LOD	< LOD	< LOD	< LOD
sludge industrial WTP	2,09	25,32	4,78	7,93	0,489	0,970	36	27
ground additive unknown (1)	<LOQ	<LOQ	4,18	<LOQ	< LOD	< LOD	< LOD	54
ground additive unknown (2)	<LOQ	<LOQ	<LOQ	<LOQ	< LOD	< LOD	< LOD	332

Phase 3 preliminary results



- **DR Calux** showed activity for several samples. Highest activity in shredder fluff, and PCB containing shredder waste
- Highest **ER Calux** activity in industrial WTP sludge, some activity also measured in ground additive and wood
- **AR Calux** activity only detected in industrial WTP sludge
- **Antagonist AR Calux** activity in several samples, however some could be false positives (cytotox)

Phase 3 preliminary results



- **Centrifugated vs non-centrifugated samples:** majority of samples show similar effects. Differences need to be double-checked

Phase 3: to be continued...



- Experiments will be repeated in a smaller range to improve quantification
- ER α Calux agonism and antagonism will be investigated and compared to ER

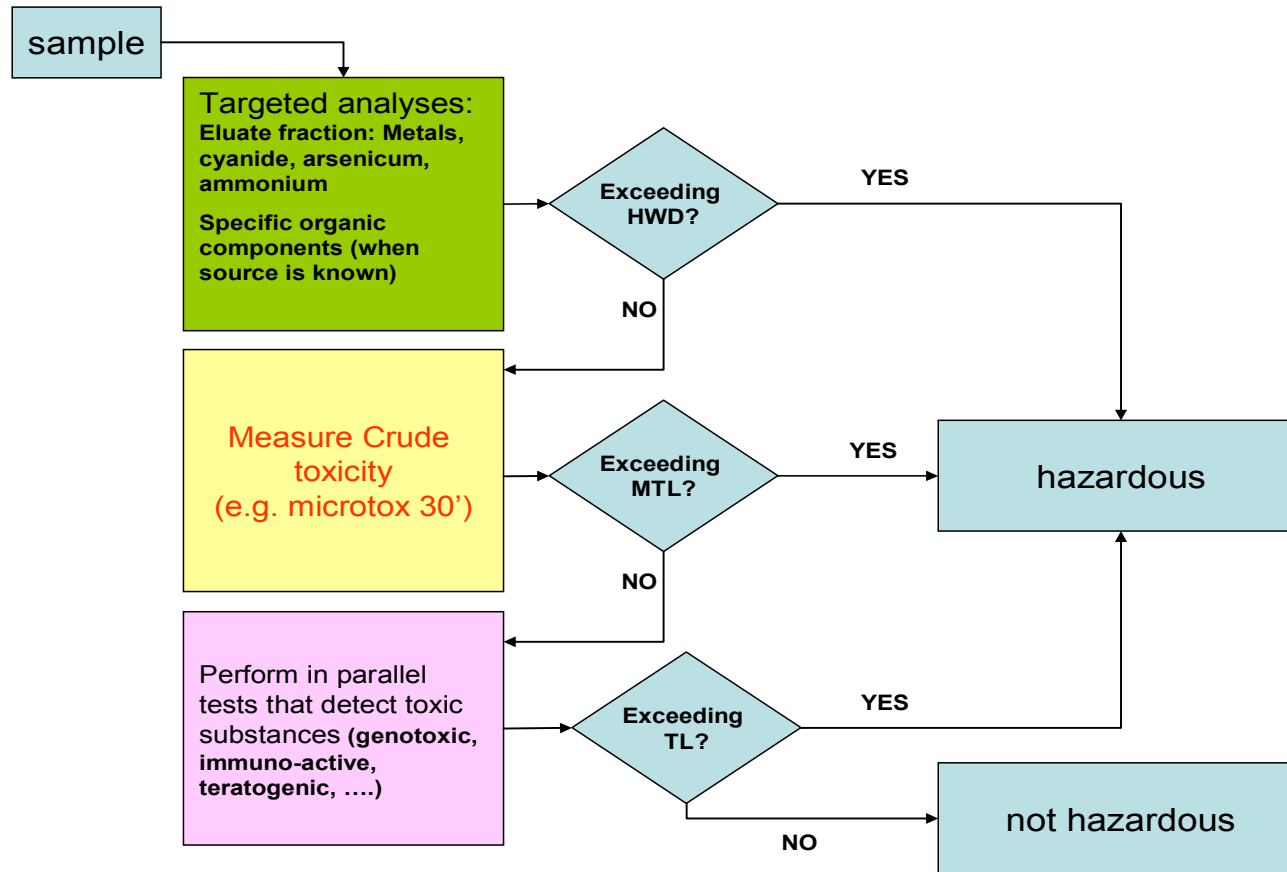
Calux

- AR Calux antagonism will further be investigated to rule out false positives (cytotox.)

conclusions



- These results show that a combination of biotests can provide a tool for hazard assessment and classification of complex waste materials.
- Bacterial test are fast and sensitive for general toxicity of the organic fraction -> use as screening – identifying extreme toxic samples (need for limit value for toxic load)
- Mechanistic assays are needed for those samples that are not extreme acute toxic





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Sander, Snezana, Hai-yen
and Roos



Luc Michiels



Johan Robbens