



In vitro genotoxicity evaluation of PAHs in mixtures using experimental design

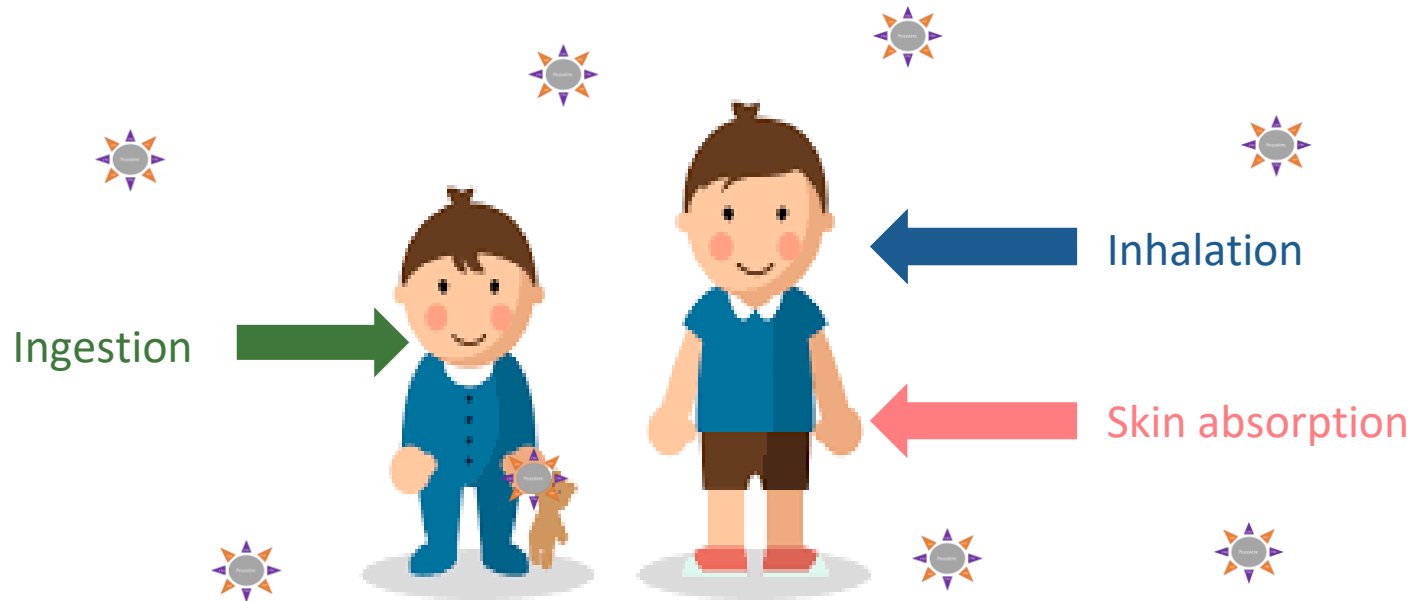
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Context



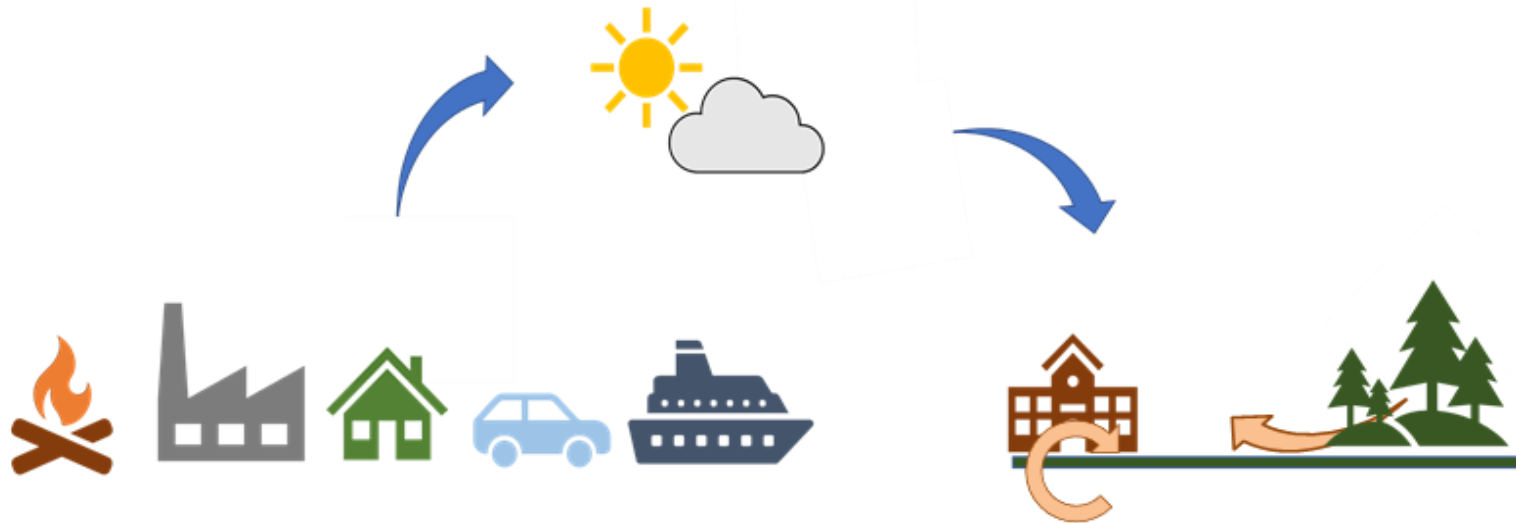
Children **below 6** years old = **sensitive population** to environmental contaminants

Specific exposure by ingestion:

Crawling, playing on the ground, bringing objects/hands to mouth

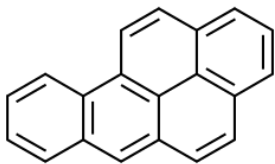
→ Up to **100 mg of dust per day** (US EPA, 2017)

Context



« **All particles settled onto objects or surfaces**, regardless of nature and origin »
(ANSES, 2019; US EPA, 2011)

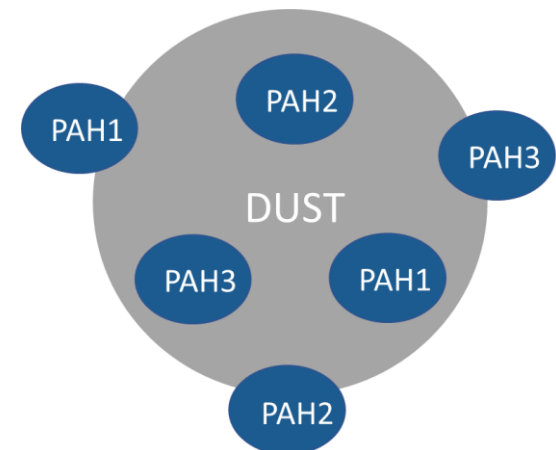
Settled dust = environmental **sink** for **organic contaminants**, especially
Polycyclic Aromatic Hydrocarbons (PAHs)



*Benzo(a)pyrene
(BaP)*

PAHs = **ubiquitous**

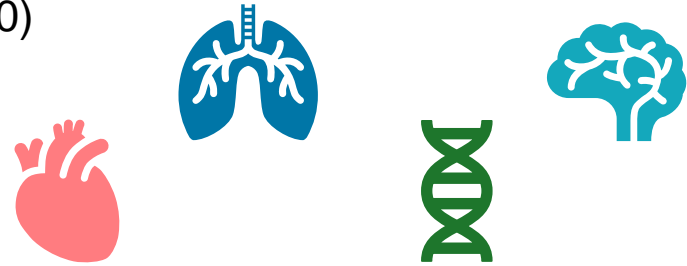
→ **16 PAHs on priority list of US EPA**



Context

Health outcomes linked to PAHs (WHO, 2010)

- Bronchitis
- Ischemic cardiopathy
- Cognitive delay
- Cancers



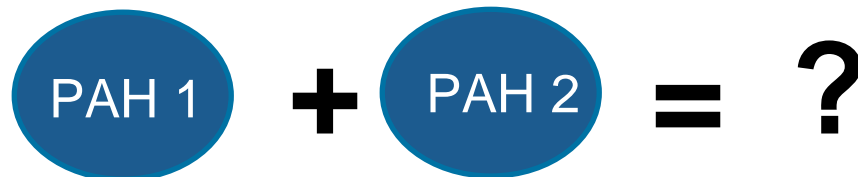
Toxic Equivalent Factors (TEFs)

Regulatory use to assess carcinogenic potential of PAH mixtures

$$TEQ = \sum (C_i \times TEF_i)$$

Little knowledge about PAHs' mixtures

Limited to binary mixtures in literature



PROPOSED TOXICITY EQUIVALENCY FACTORS (TEFS) FOR INDIVIDUAL PAHS

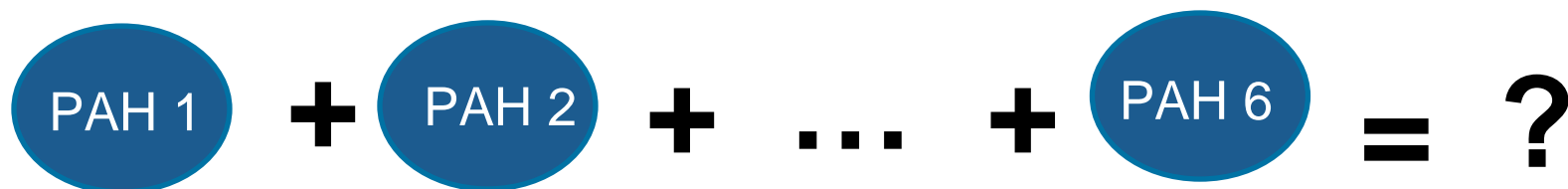
Compound	TEF
Dibenzo[<i>a,h</i>]anthracene	5 ^a
Benzo[<i>a</i>]pyrene	1
Benzo[<i>a</i>]anthracene	0.1
Benzo[<i>b</i>]fluoranthene	0.1
Benzo[<i>k</i>]fluoranthene	0.1
Indeno[123- <i>c,d</i>]pyrene	0.1
Anthracene	0.01
Benzo[<i>g,h,i</i>]perylene	0.01
Chrysene	0.01
Acenaphthene	0.001
Acenaphthylene	0.001
Fluoranthene	0.001
Fluorene	0.001
2-Methylnaphthalene	0.001
Naphthalene	0.001
Phenanthrene	0.001
Pyrene	0.001

Scientific question:

Are the effects of PAHs additive, when adjusted by their TEFs and when in mixture?

Aims

1. Investigate binary interaction effects of 6 PAHs in mixtures with *in vitro* assays
2. Examine whether the TEFs can roughly predict the *in vitro* DNA damage & chromosomal damage

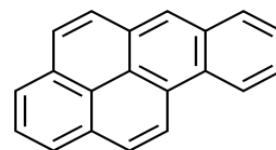


Methodology

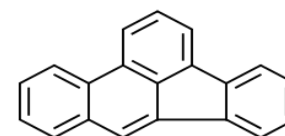
- Use of experimental design = maximize useful data with minimal number of experiments

- 6 PAHs for mixtures

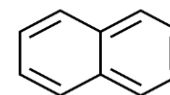
- Selection based on **carcinogenicity** / environmental distribution / **source linked to a specific activity**



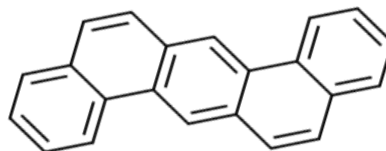
Benzo(a)pyrene
(BaP); IARC: 1



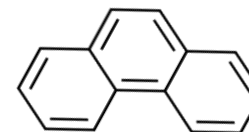
Benzo(b)fluoranthene
(BbF); IARC: 2B



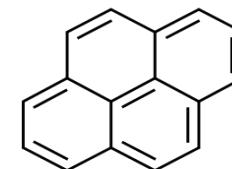
Naphthalene
(Nap); IARC: 2B



Dibenz(a,h)anthracene
(dBahA); IARC: 2A



Phenanthrene
(Phe); IARC: 3

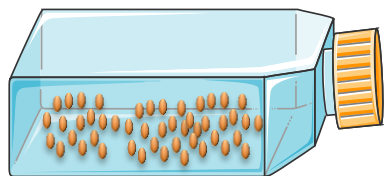


Pyrene
(Pyr); IARC: 3

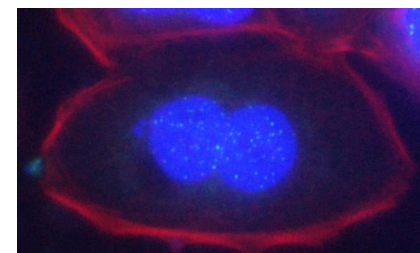
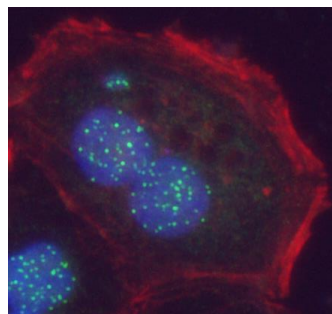
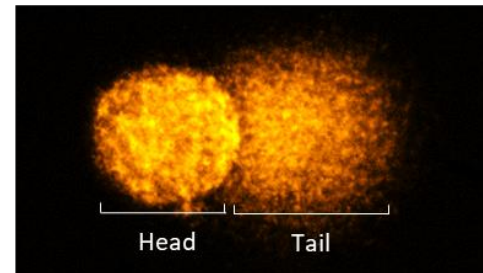
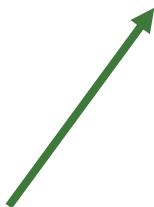
Methodology

Alkaline comet assay –
DNA damage (Tice *et al.*, 2000)

4h exposure to mixtures
+ 10% S9 mix



Adenoma Gastric Cells
(AGS), passage #42-43
Doubling time = 20h



Cytokinesis Block Micronucleus assay–
Chromosomal damage (Fenech, 2007)

Methodology: design of experiment

Experiment	Nap	Phe	Pyr	BbF	BaP	dBahA	TEQ
1	1	1	1	0,1	0,0025	0,1	0,116
2	1	10	10	1	1	1	2,121
3	10	1	10	1	1	1	2,121
4	10	10	1	1	1	1	2,121
5	10	10	10	0,1	1	1	2,04
6	10	10	10	1	0,0025	1	1,133
7	10	10	10	1	1	0,1	1,23
8	10	10	1	0,1	0,0025	0,1	0,134
9	10	1	10	0,1	0,0025	0,1	0,134
10	10	1	1	1	0,0025	0,1	0,215
11	10	1	1	0,1	1	0,1	1,122
12	10	1	1	0,1	0,0025	1	1,025
13	1	10	10	0,1	0,0025	0,1	0,134
14	1	10	1	1	0,0025	0,1	0,215
15	1	10	1	0,1	1	0,1	1,122
16	1	10	1	0,1	0,0025	1	1,025
17	1	1	10	1	0,0025	0,1	0,215
18	1	1	10	0,1	1	0,1	1,122
19	1	1	10	0,1	0,0025	1	1,025
20	1	1	1	1	1	0,1	1,203
21	1	1	1	1	0,0025	1	1,106
22	1	1	1	0,1	1	1	2,013
TEF	0,001	0,001	0,001	0,1	1	1	

$$TEQ = \sum (C_i \times TEF_i)$$

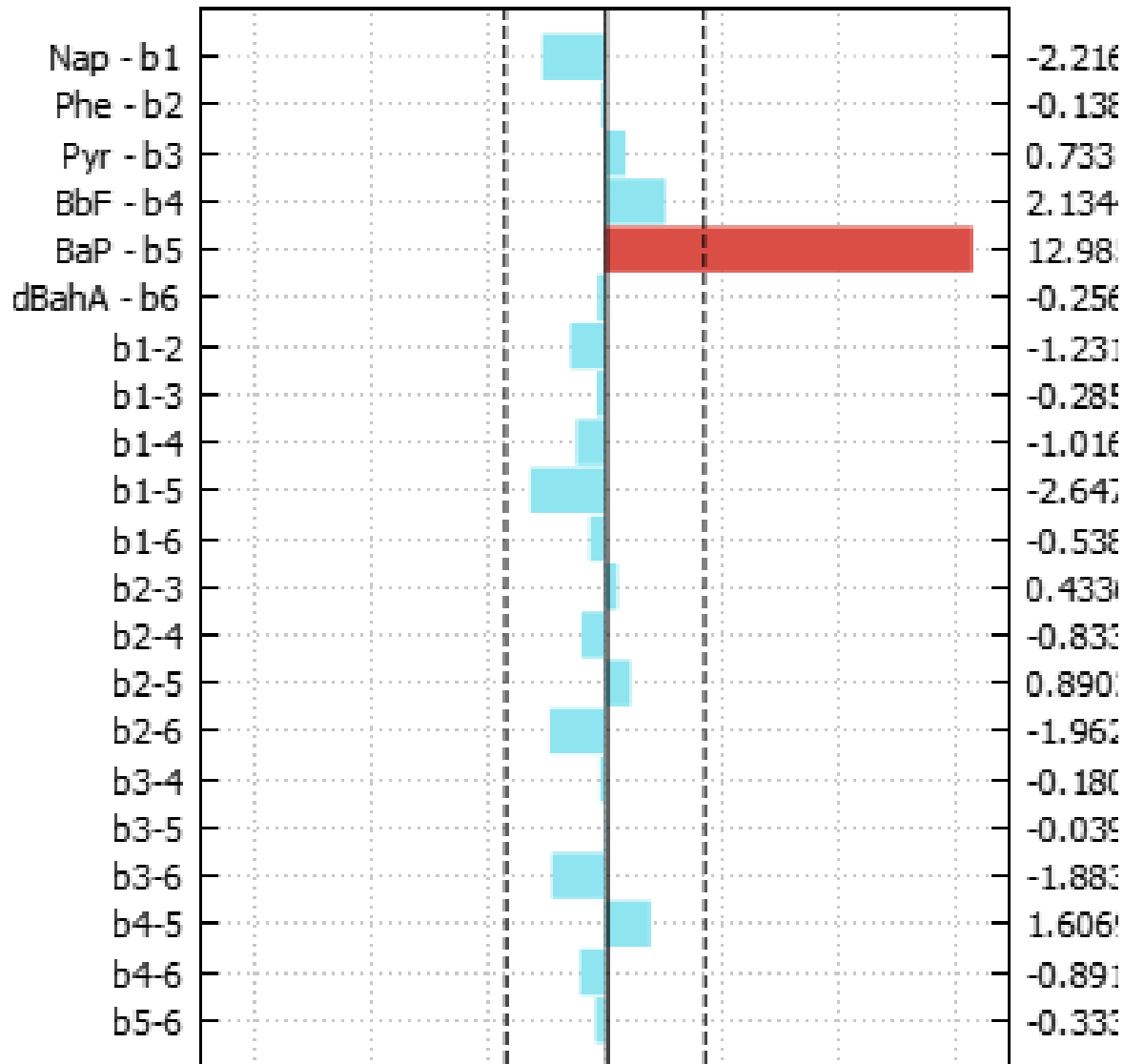
Alkaline comet assay
experimental design

Methodology: design of experiment

Experiment	Nap	Phe	Pyr	BbF	BaP	dBahA	TEQ
1	1	1	1	0.2	0.0025	0.2	0.2255
2	1	10	10	2	2	2	4.221
3	10	1	10	2	2	2	4.221
4	10	10	1	2	2	2	4.221
5	10	10	10	0.2	2	2	4.05
6	10	10	10	2	0.0025	2	2.2325
7	10	10	10	2	2	0.2	2.43
8	10	10	1	0.2	0.0025	0.2	0.2435
9	10	1	10	0.2	0.0025	0.2	0.2435
10	10	1	1	2	0.0025	0.2	0.4145
11	10	1	1	0.2	2	0.2	2.232
12	10	1	1	0.2	0.0025	2	2.0345
13	1	10	10	0.2	0.0025	0.2	0.2435
14	1	10	1	2	0.0025	0.2	0.4145
15	1	10	1	0.2	2	0.2	2.232
16	1	10	1	0.2	0.0025	2	2.0345
17	1	1	10	2	0.0025	0.2	0.4145
18	1	1	10	0.2	2	0.2	2.232
19	1	1	10	0.2	0.0025	2	2.0345
20	1	1	1	2	2	0.2	2.403
21	1	1	1	2	0.0025	2	2.2055
22	1	1	1	0.2	2	2	4.023
TEF	0.001	0.001	0.001	0.1	1	1	

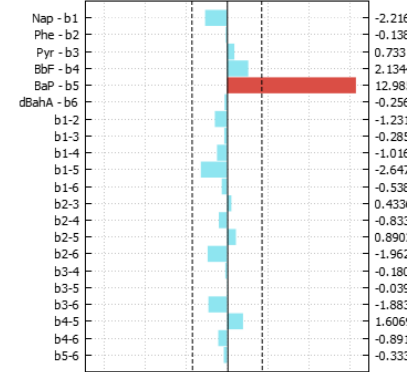
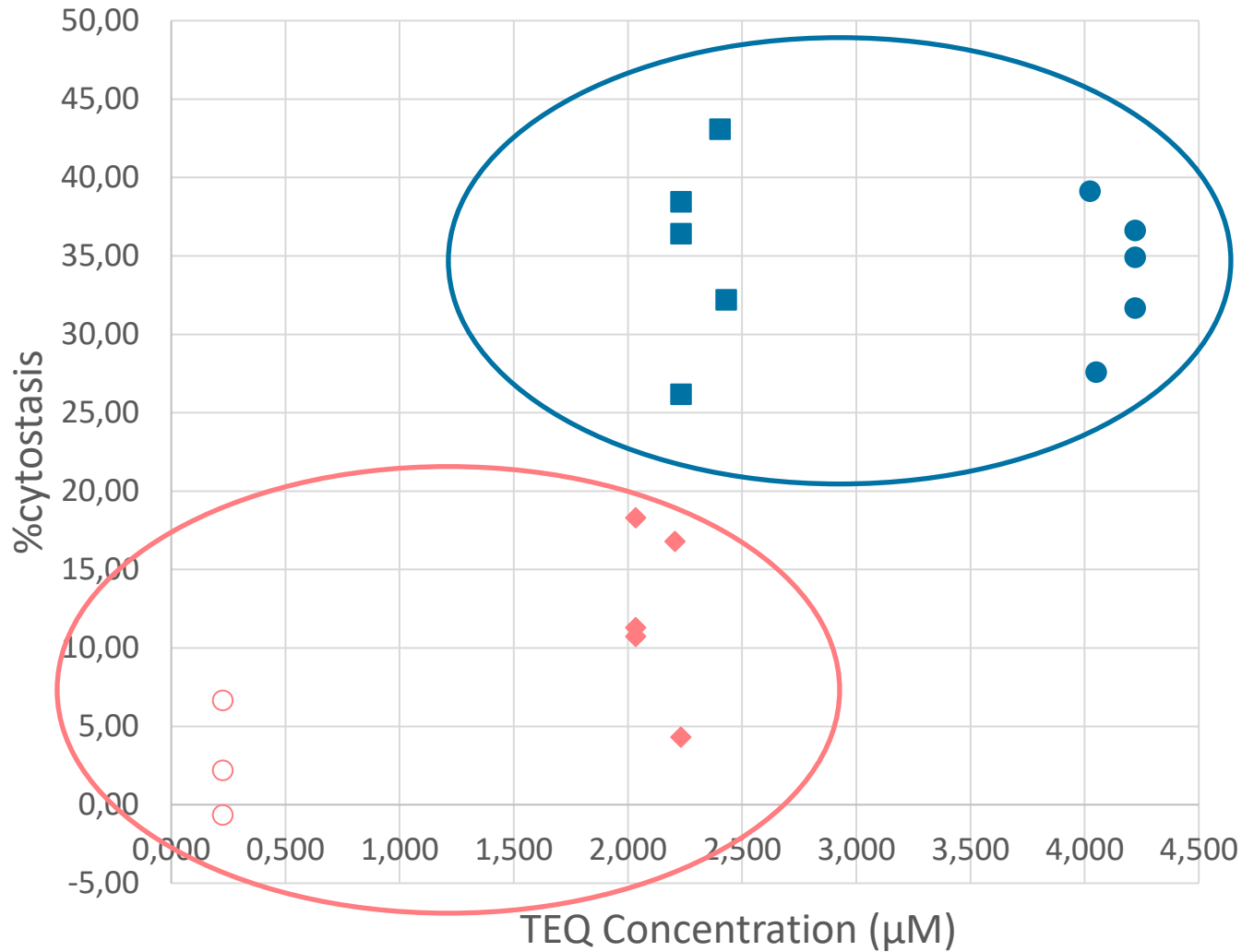
Cytokinesis Block Micronucleus (CBMN) assay experimental design

Results: Binary interactions - Cytostasis



→ BaP = only factor with effect on cytostasis

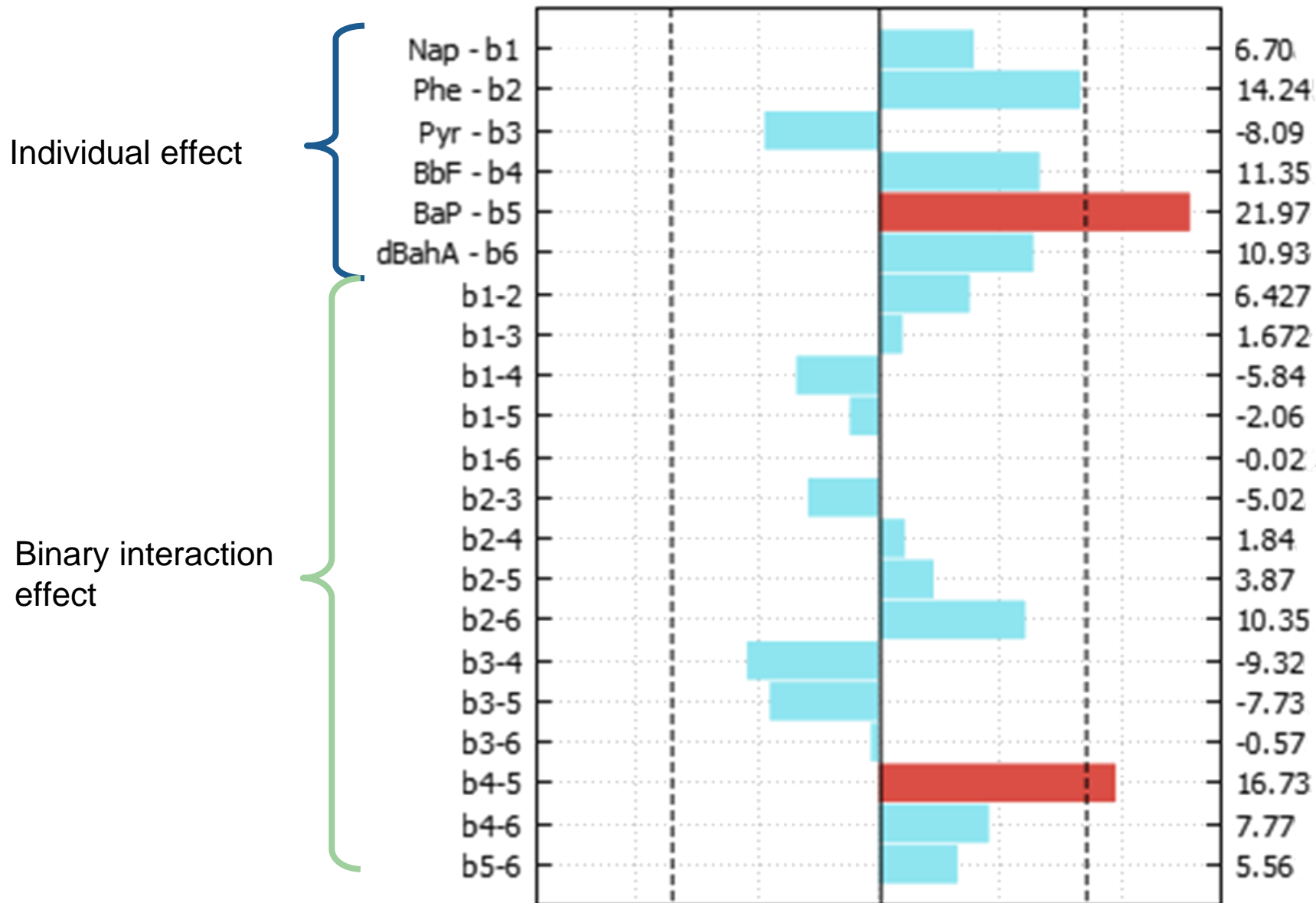
Results: Binary interactions - Cytostasis



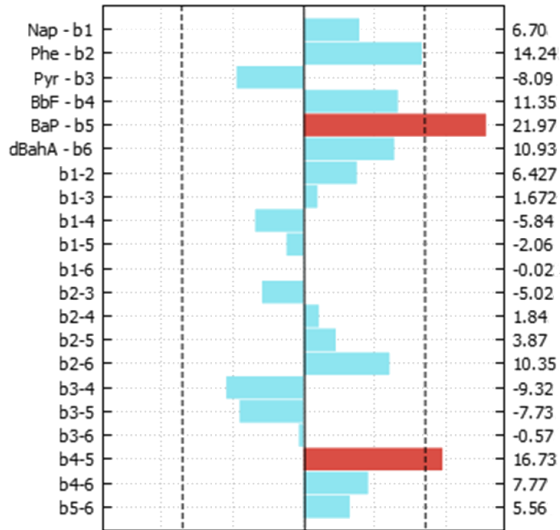
- BaP + / dBahA -
- BaP + / dBahA +
- ◆ BaP - / dBahA +
- BaP - / dBahA -

→ BaP = only factor with effect on cytostasis

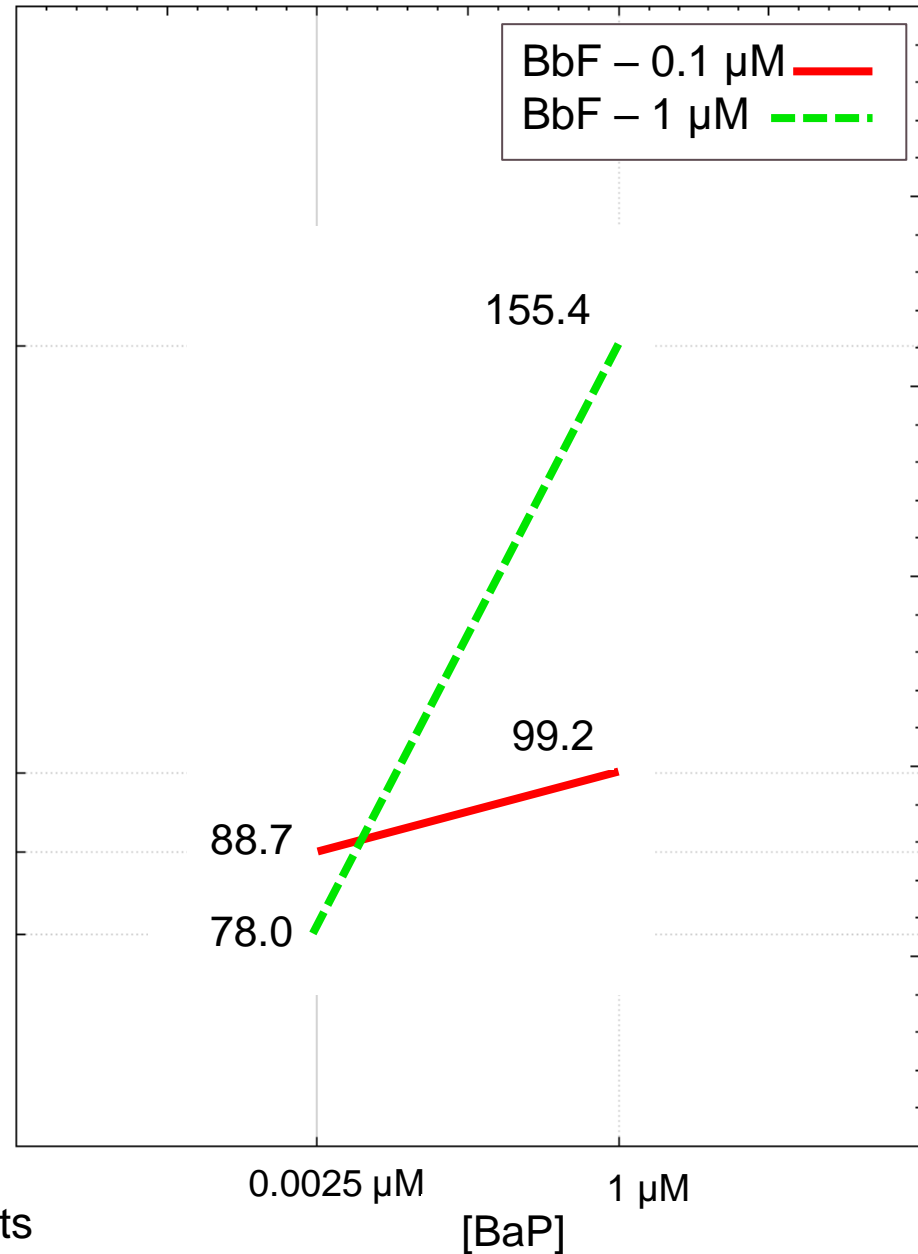
Results: Binary interactions – comet assay



Results: Binary interactions – comet assay



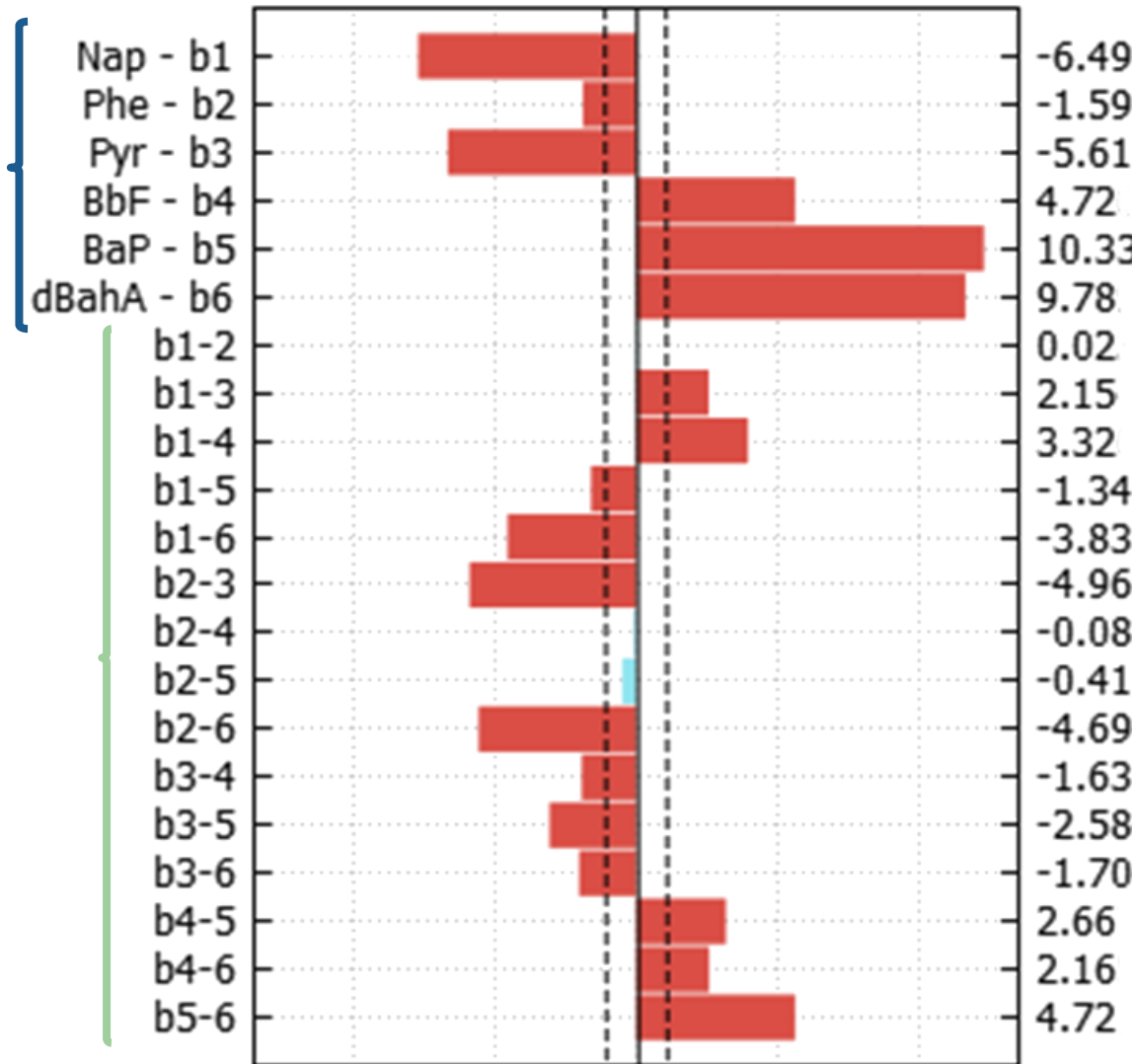
% increase TailDNA



→ Synergistic interaction

Tarantini *et al.*, 2011 → DNA adducts

Results: binary interactions – CBMN assay



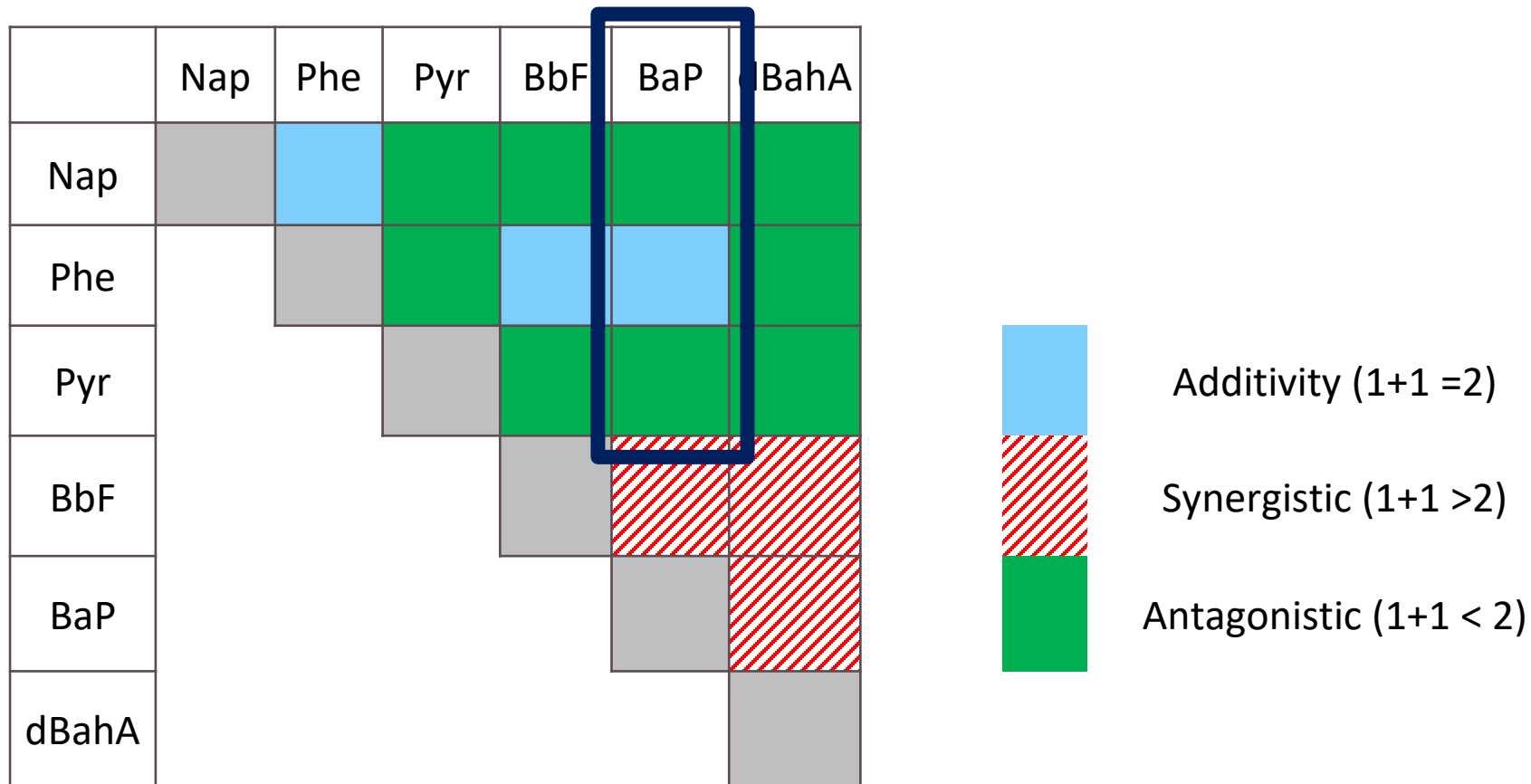
Many significant binary interactions effects

Most important are:

- b2-3: Phe – Pyr
- b2-6: Phe – dBahA
- b5-6: BaP – dBahA

X2 value

Results: Binary interactions matrix – CBMN assay



Muthusamy *et al.*, 2018: *in vitro* flow cytometry micronucleus assay

- BaP + Nap = antagonistic
- BaP + Phe = antagonistic
- BaP + Pyr = additive

Results: Determination of Genotoxic Equivalent Factors (GEF)



Single PAH		
PAH	TEF	GEF (γ H ₂ AX)
Nap	0.001	0
Phe	0.001	0
Pyr	0.001	0
BbF	0.1	[0.69-1.46]
dBahA	1	[0.5-2.77]
BaP	1	1.00

Tomasetig *et al.*, 2020

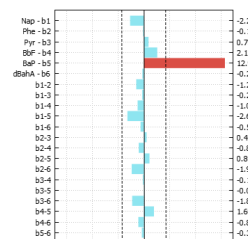
Determination of scaled coefficients with a least square regression without intercept model

Conclusion

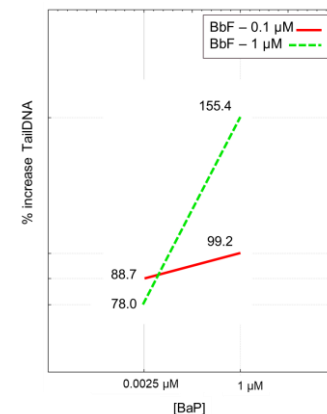
- Experimental designs = binary interaction effects of 6 PAHs in mixtures
- *In vitro* comet assay & CBMN assay on AGS cells
- Determination of GEFs & comparison to TEFs

• Main results

- No interaction for cytostasis
- Comet assay: synergistic interaction BaP – BbF
- CBMN: many interactions
- GEFs consistent with TEFs



	Nap	Phe	Pyr	BbF	BaP	dBahA
Nap	Grey	Blue	Green	Green	Green	Green
Phe		Grey	Green	Blue	Green	Green
Pyr			Grey	Green	Green	Green
BbF				Grey	Red	Red
BaP					Grey	Red
dBahA						Grey



Future prospects

GEF for PAHs : more testing necessary of PAHs in mixtures with other genotoxicity assays and other cell lines

Experimental designs = optimized method to study contaminants' interactions in mixtures → useful approach

Further study of interactions

- Ternary and other levels of interactions
- Different contaminants in mixtures: inorganic / organic

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Thank you for your attention!

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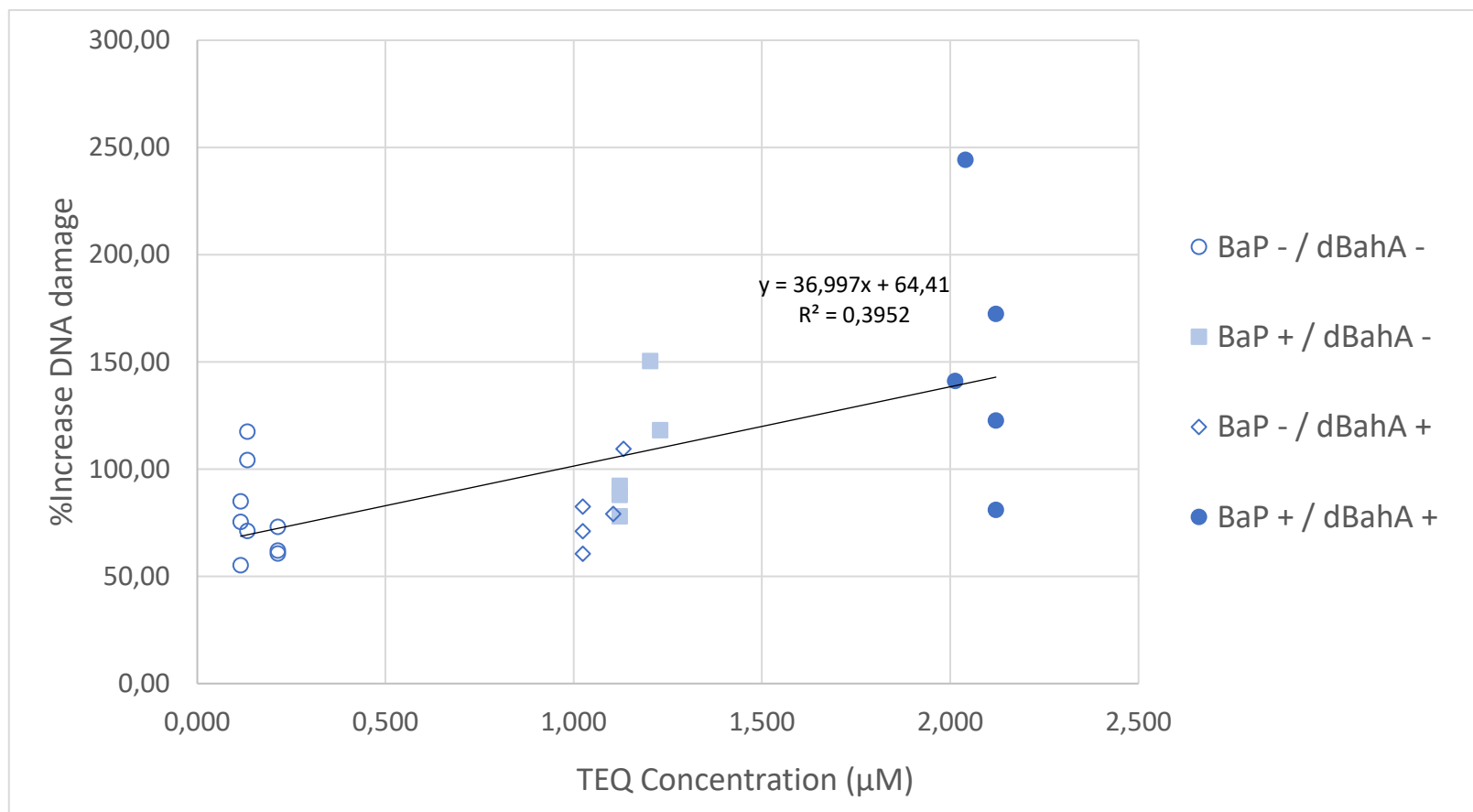
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Annex: Methodology

- Statistical analysis for binary interactions
 - Comet assay: Kruskal-Wallis multiple comparison test
 - CBMN: chi-2 test
 - Experimental design: Student test
- PAH behavior in mixtures and TEF
 - Linear regression coefficients without intercept calculated for each PAH
 - Scaling on BaP values and comparison

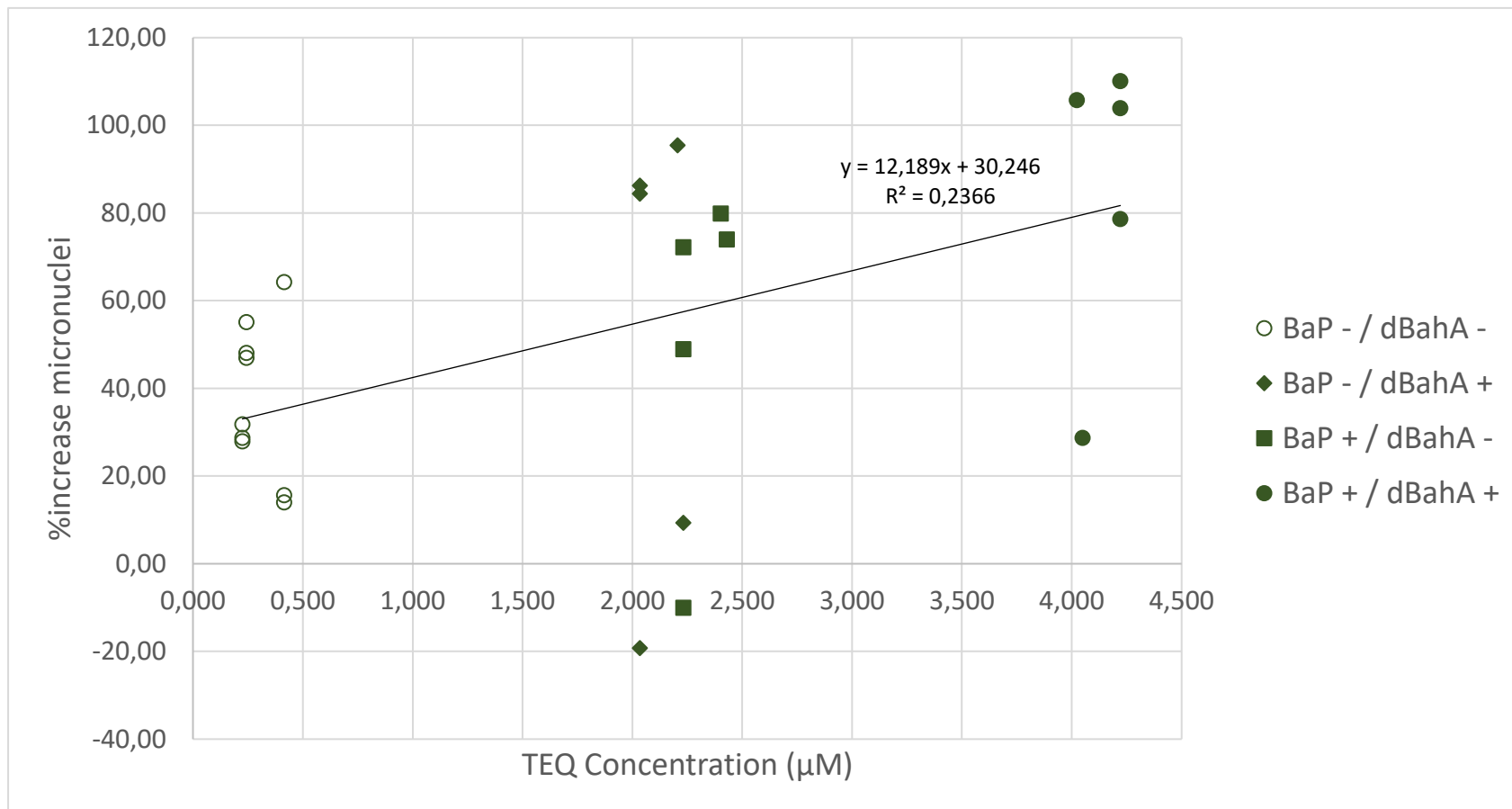
Results

- TEQ and DNA damage



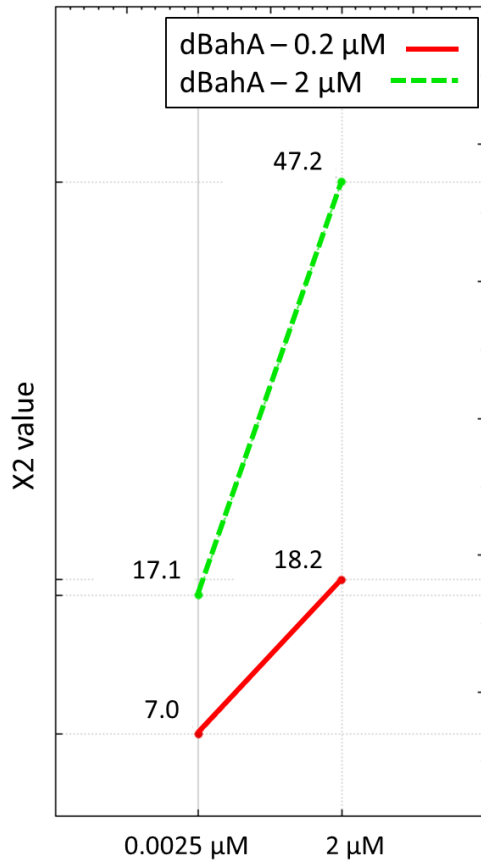
Results

- TEQ and micronuclei

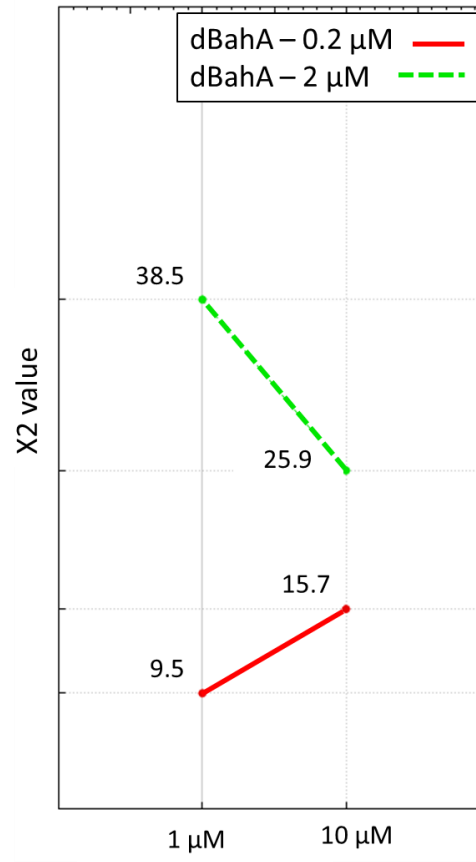


Results

- Binary interactions – CBMN assay



Synergistic



Antagonistic

