ALTERNATIVES FOR INHALATION TOXICITY TESTING CASE STUDIES (VITROCELL® 6/4 & 24/48)

WebEx, April 30th

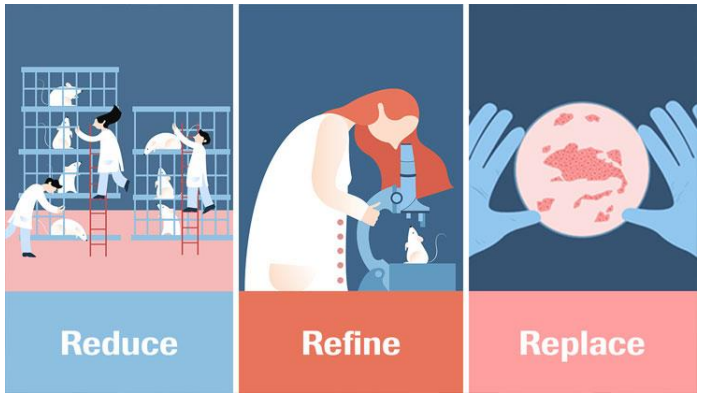
FLEMISH INSTITUTE FOR TECHNOLOGICAL RESEARCH

- Strategic research center of Flanders
- Funded (1/3) by the Flemish Government
- Headquarter located in Mol, Belgium

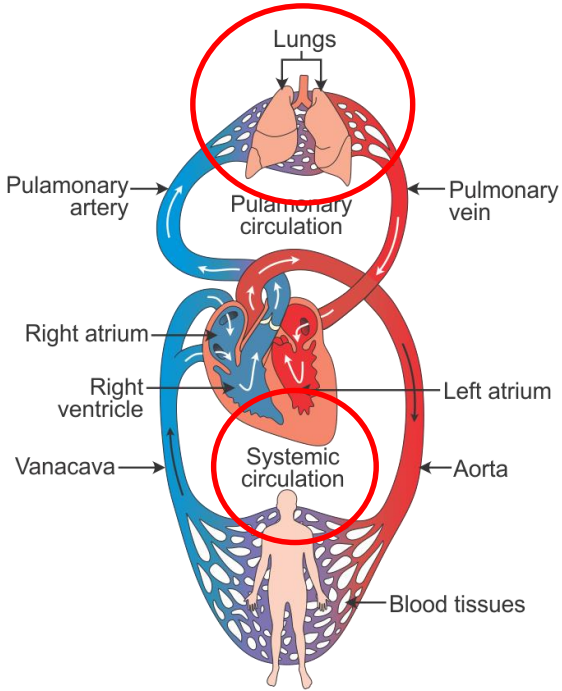
INHALATION TOXICITY TESTING

BACKGROUND

Picture from PETA

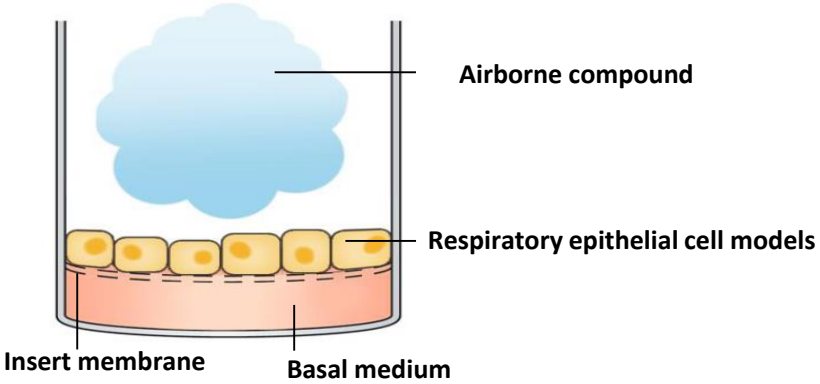


Picture from Roche



NEED FOR ALTERNATIVES

IN VITRO AIR-LIQUID INTERFACE (ALI) EXPOSURE

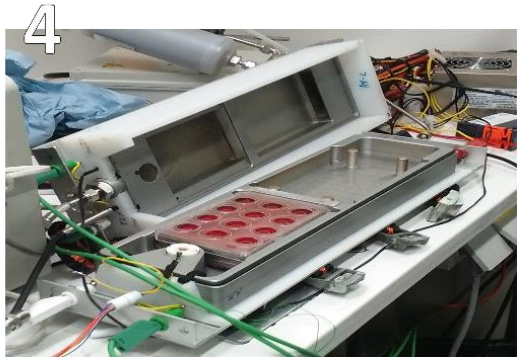


Relevant respiratory cell models
Realistic inhalation exposure systems
Proper dosimetry techniques

4 ALI EXPOSURE SYSTEMS

1. **VITROCELL® 6/4 module**
   - Electrostatic depositor
   - 4 replicates
2. **VITROCELL® 24/48 module**
   - Simultaneous exposure negative, positive control, 6 concentrations compound
   - 6 replicates for each condition
3. **VITROCELL® Cloud 12 module**
   - 3 replicates control, 8 replicates compound, 1 microbalance
4. **NAVETTA**
   - Patented in-house co-developed module (Frijns *et al.*, 2017)
   - 4 replicates

Adapters 12- and 24-well sizes / stainless steel inserts



CASE 1: SILANE COMPOUNDS

GOAL

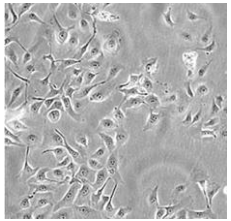
- *In vitro* approaches for assessing respiratory toxicity of chemicals would provide useful information to product development and risk management decisions

- **INSPiRE** project: demonstrate utility of an *in vitro* system
  - to predict the likelihood of a chemical to cause effects on the human respiratory tract
  - to rank chemical toxicity

CASE 1A: TRIETHOXYSILANE

- Demonstrate performance of *in vitro* system using triethoxysilane (TES)

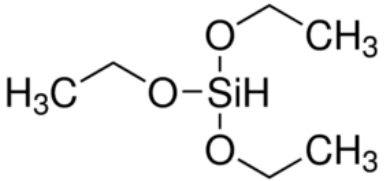
✓Optimization of TES vapor generation
✓Generation of TES vapor and dry exposure of lung cells


BEAS-2B cells


VITROCELL 6/4®

TES

- Industrial chemical
- GHS category 2 inhalation toxicant (~rat acute inhalation toxicity testing)
- Not stable, highly reactive with water



OPTIMIZATION OF TES VAPOR GENERATION

- TES generation in dry clean air using a capillary dosage system (Goelen *et al.* 1992)
- Stability of compounds monitored online by GC-FID (gas chromatography-flame ionization detector) and THC (total hydrocarbon) analyzer

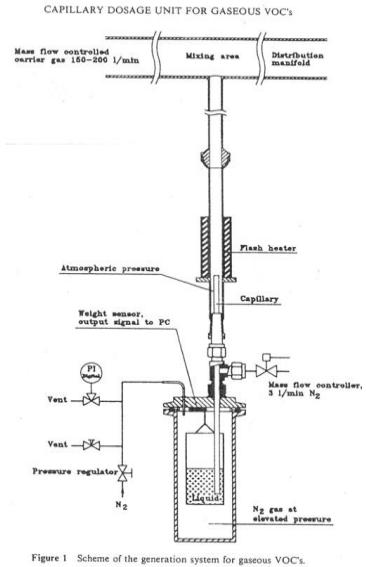


Figure 1 Scheme of the generation system for gaseous VOC's.

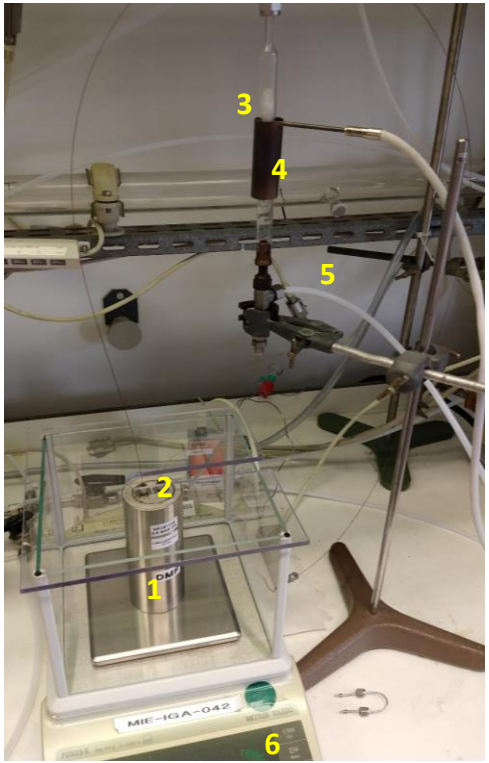
OPTIMIZATION OF TES VAPOR GENERATION

1. Recipient with TES on analytical balance
2. Pressure on closed recipient
3. Outgoing liquid retained by cotton plug
4. Liquid evaporated by local heating element
5. TES vapor transferred by dry air flow to glass distribution line
6. Weight loss monitored

Stability monitored by GC-FID and THC analyzer

Different concentrations obtained by altering weight loss and air flows (>6 ppm) and use of measured value of THC analyzer (<6 ppm)

Splitter mass flow controller needed for concentrations <6 ppm



GENERATION OF TES VAPOR AND DRY EXPOSURE OF LUNG CELLS – STUDY DESIGN

ALI exposure system	Capillary dosage unit coupled to VITROCELL® 6/4
Respiratory cell model	BEAS-2B (*normal* human bronchial epithelial cell line)
Type of inserts	Precoated Corning® Transwell® polyester membrane inserts (Sigma-Aldrich), pore size 0.4 µm, diameter 24 mm (6-well)
Seeding density on inserts	15000 cells/cm^2
Growth protocol	48 hours (h) submerged growth, exchange BEGM for BEBM +/- 16 h prior ALI exposure
Flow/insert	3 millilitre per minute (mlpm)
Nozzle height	3 mm
Conditioning	Temperature during exposure: 37 °C Dry exposure because of reactivity TES

BEGM: Bronchial Epithelial Growth Medium (with supplements)
BEBM: Bronchial Epithelial Basal Medium
Coating: 10 µg/ml human plasma fibronectin, 30 µg/ml PureCol™, and 10 µg/ml bovine serum albumin dissolved in BEBM

GENERATION OF TES VAPOR AND DRY EXPOSURE OF LUNG CELLS – STUDY DESIGN

Concentrations test conditions	• Incubator control (**IC**): inserts without apical medium kept in the incubator for 24 h, as control for clean air (CA) • **CA** • Positive control: 12 ppm nitrogen dioxide (**NO_2**) • Lipopolysaccharide (**LPS**): submerged exposure to 20 µg/ml as positive control for cytokine secretion • **TES** vapor: 0.72, 25, 85 ppm (based on LC50 data: >500 and < 1300 mg/m^3)
Exposure time	1 h
Submerged post-exposure time	20-24 h for cell viability/inflammation; 30 minutes (min) for cytotoxicity
Biological endpoints (and assays)	Cell viability (PrestoBlue™) Cytotoxicity (lactate dehydrogenase, LDH) Inflammation: IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, IFN-β, TNF-α (Meso Scale Discovery, V-PLEX)

GENERATION OF TES VAPOR AND DRY EXPOSURE OF LUNG CELLS – STUDY DESIGN

Calculating delivered/cellular dose	ICP-AES (inductively coupled plasma-atomic emission spectroscopy) Silicium standard in 5% TMAH (tetramethylammoniumhydroxide); digestion in TMAH
Replicates/run	4
Runs	3 independent biological experiments
Existing *in vivo* data?	• **Acute inhalation toxicity – GHS category 2** • Acute oral toxicity – GHS category 4 • Skin irritation – GHS category 2 • Eye irritation – GHS category 1
Known Mode of Action (key events)	• Cellular absorption and hydrolysis of TES • Cell death • Loss of epithelial barrier • Secretion of inflammatory cytokines • Pulmonary oedema / hemorrhage

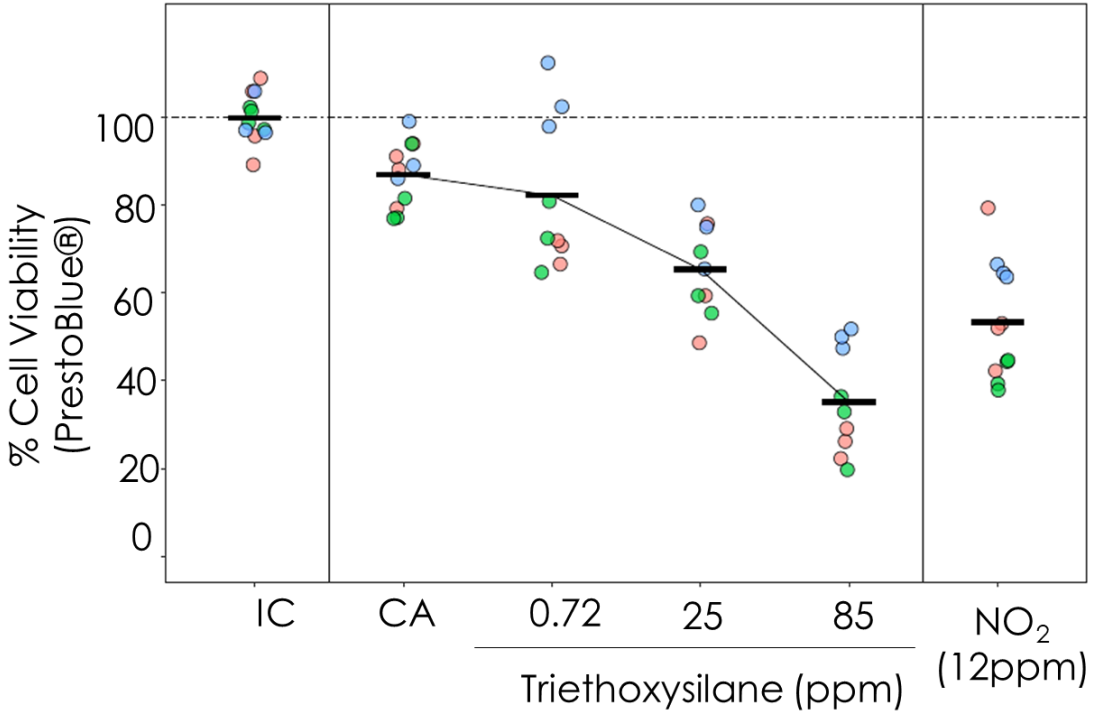
DELIVERED CONCENTRATION (ICP-AES, DIRECT)

Exposure concentration TES (ppm)	Concentration of TES in **cells** (µg)			
	N=1	N=2	N=3	Avg
0.72	/	/	/	/
25	2.6	3.3	3.8	3.2
85	15.4	17.4	20.8	17.9

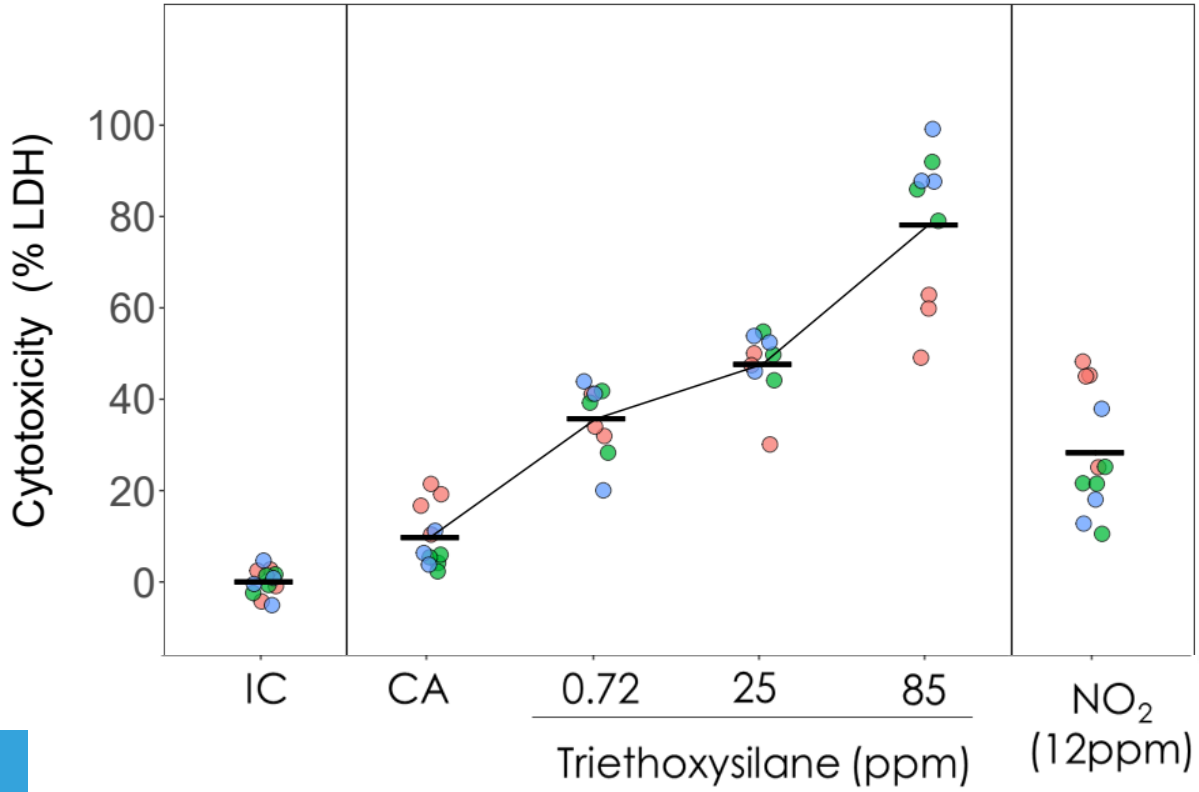
Delivery efficiency: 14%

Exposure concentration TES (ppm)	Concentration of TES in **medium** (µg)			
	N=1	N=2	N=3	Avg
0.72	/	/	/	/
25	2.8	2.5	2.3	2.5
85	5.9	6.3	5.8	6.0

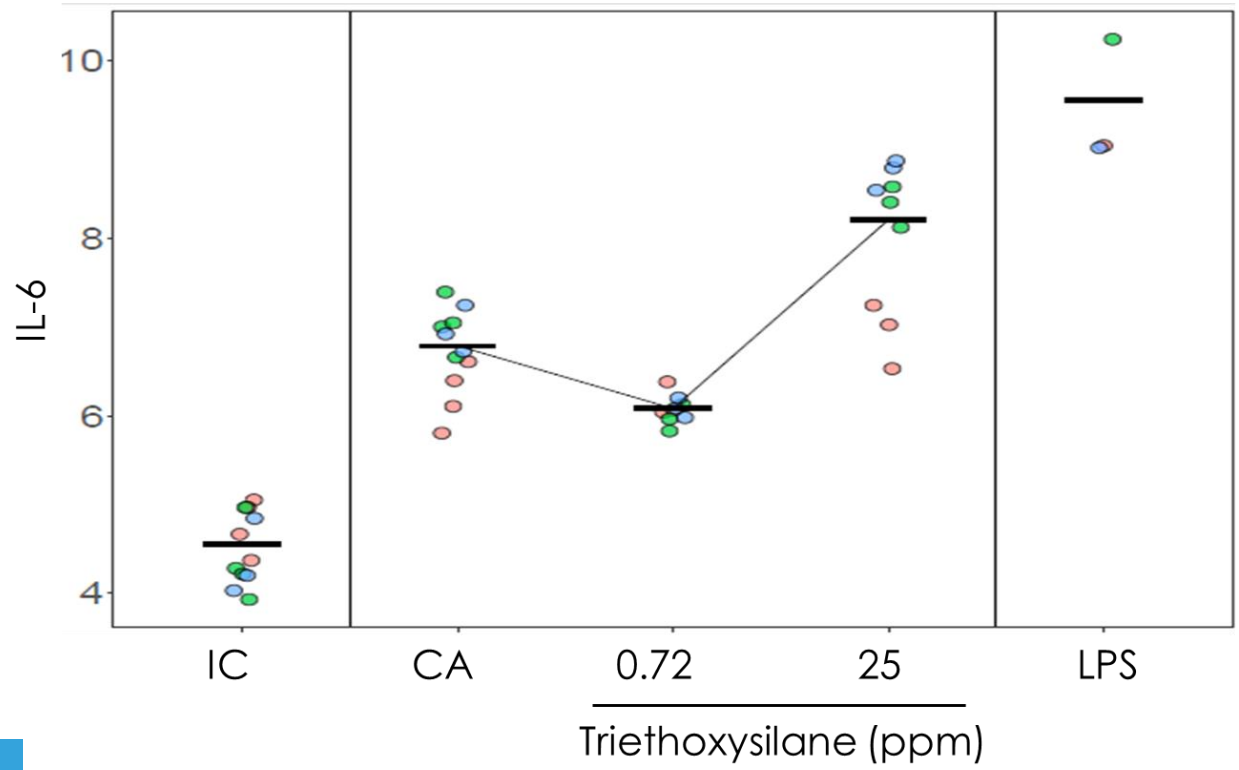
CELL VIABILITY (PRESTOBLUE™, 20-24 H POST-EXPOSURE)



CYTOTOXICITY (LDH ASSAY, 30 MIN POST-EXPOSURE)



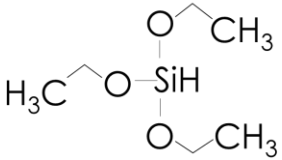
INFLAMMATION (CYTOKINE RELEASE, 20-24 H POST-EXPOSURE, MSD)



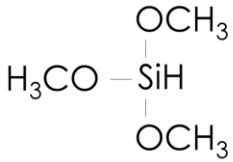
Same trend for
IL-2
IL-4
IL-8
IL-10
IL-12p70
IL-13
TNF-α

NEXT STEPS 2020

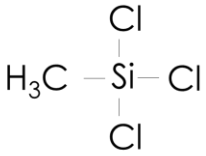
- Additional work is underway to test other silane compounds
- Determine if this *in vitro* system can detect the decrease in toxicity that correlates with increasing carbon length



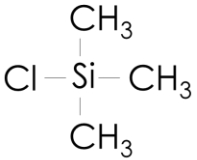
Triethoxysilane
Inhalation toxicity:
GHS 2



Trimethoxysilane
Inhalation toxicity:
GHS 1



Methyltrichlorosilane
Inhalation toxicity:
GHS 3



Trimethylchlorosilane
Inhalation toxicity:
GHS 3

- Determine advantages of using a 2D cell line (BEAS-2B) versus a 3D human reconstructed tissue model

BACKGROUND

- PETRALI project

- Subject to regulatory registration requirements!

BUT

- Volatile to semi-volatile
- Low aqueous solubility

AND

- Many individual constituents with a range of different physicochemical properties

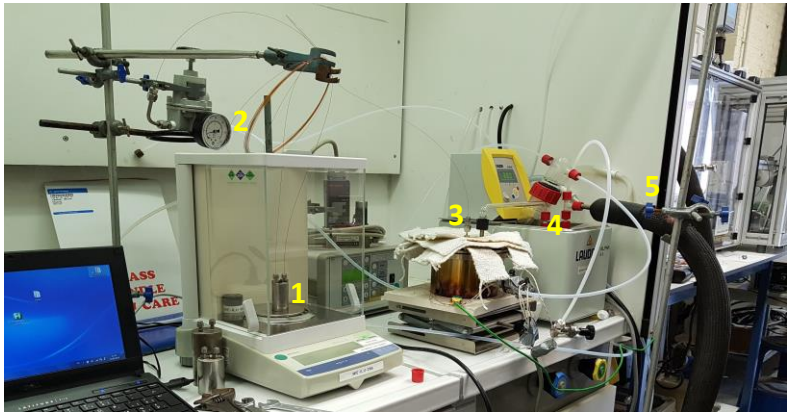
SO

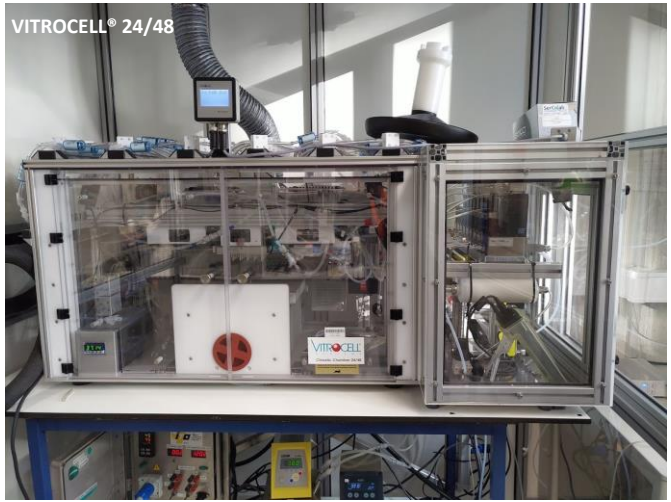
- Difficult to test '*in vitro*' for inhalation toxicity

GOAL

- Develop alternative method and replace *in vivo* animal tests for prediction of human *in vivo* inhalation toxicity

✓Development and validation of a generation facility to obtain vapors
✓Optimization & validation of an ALI exposure system



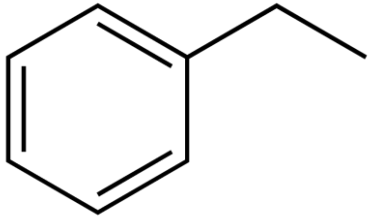


SINGLE COMPOUND TESTING

- Demonstrate performance of ALI exposure method using EB

EB

- Mono-aromatic hydrocarbon constituent of petroleum
- Occupational exposure during refinery operations
- H304 and H332
- DNEL



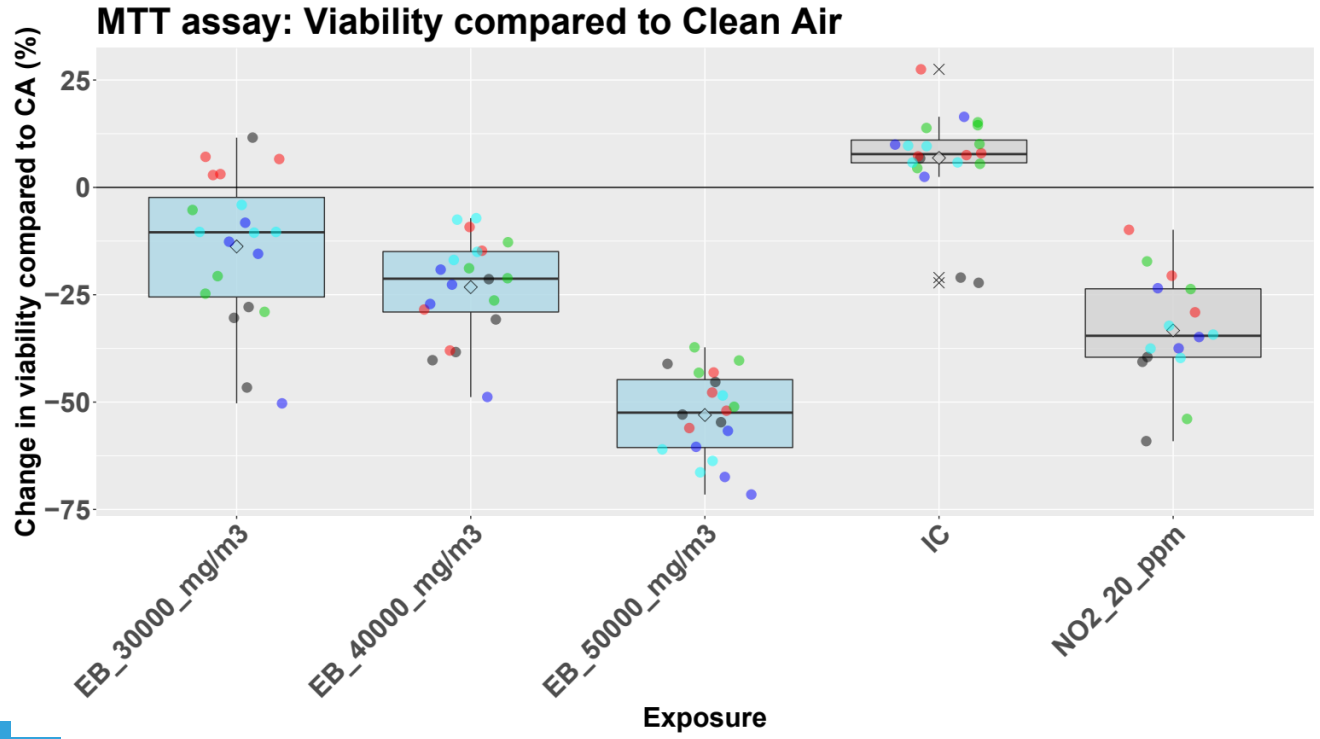
Picture from ExxonMobil

HEADSPACE GC-MS (STAINLESS STEEL INSERTS)

Avg. measured EB exposure concentration +/- STDEV (mg/m^3)	Avg. delivered EB dose +/- STDEV (µg)
51562 +/- 228	22.7 +/- 3.5
40989 +/- 181	14.3 +/- 4.3
30989 +/- 137	9.1 +/- 2.9

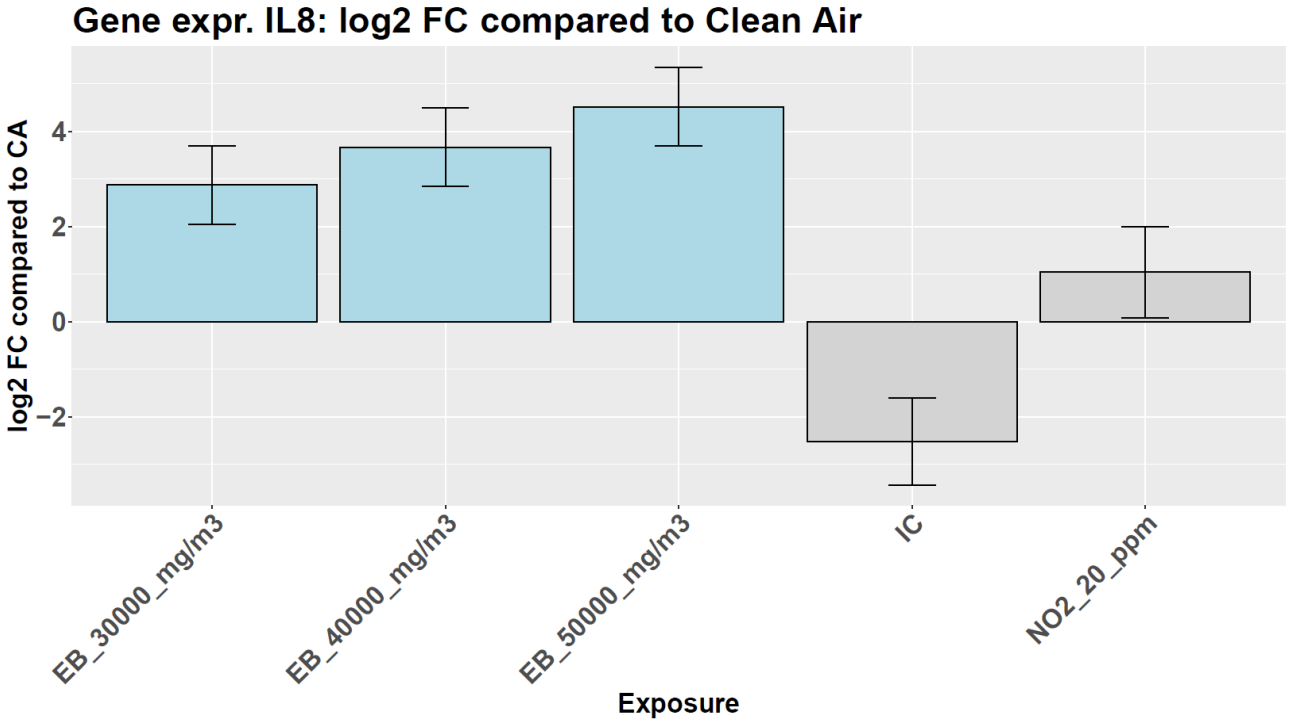
Delivery efficiency: 0.1%

CELL VIABILITY



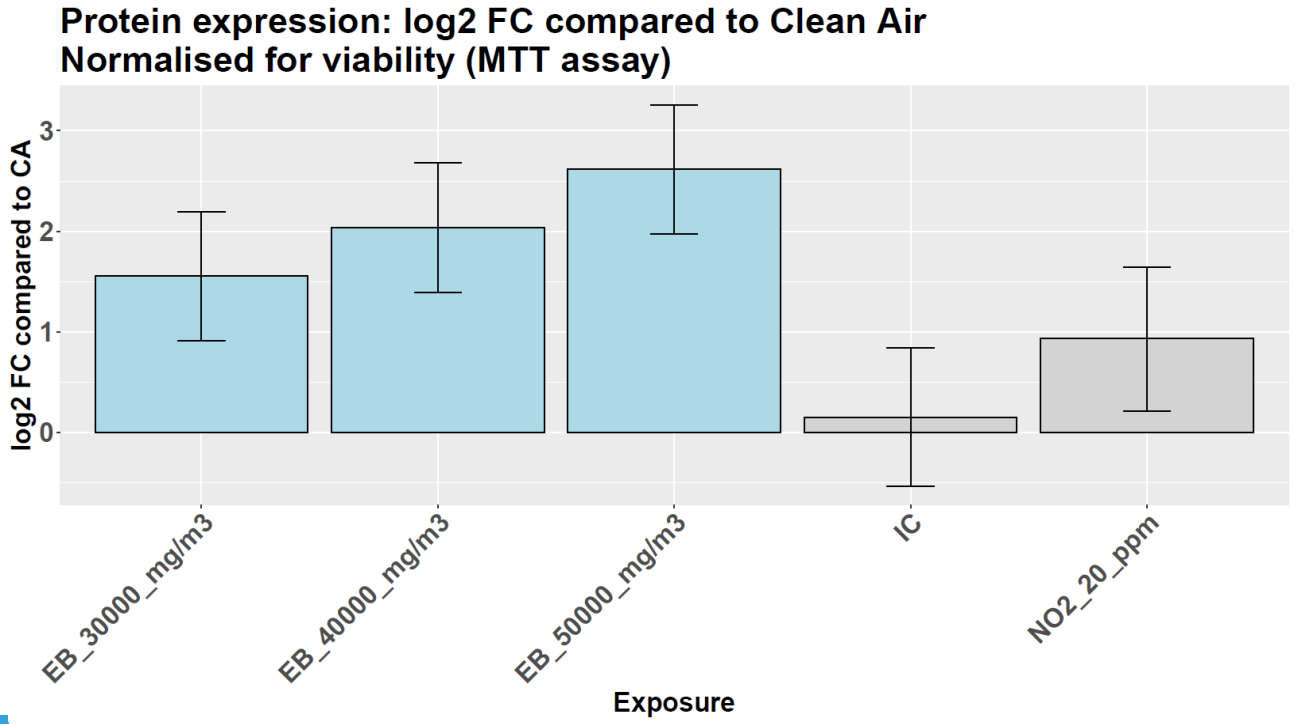
Confirmed in LDH assay

INFLAMMATION (GENE EXPRESSION)

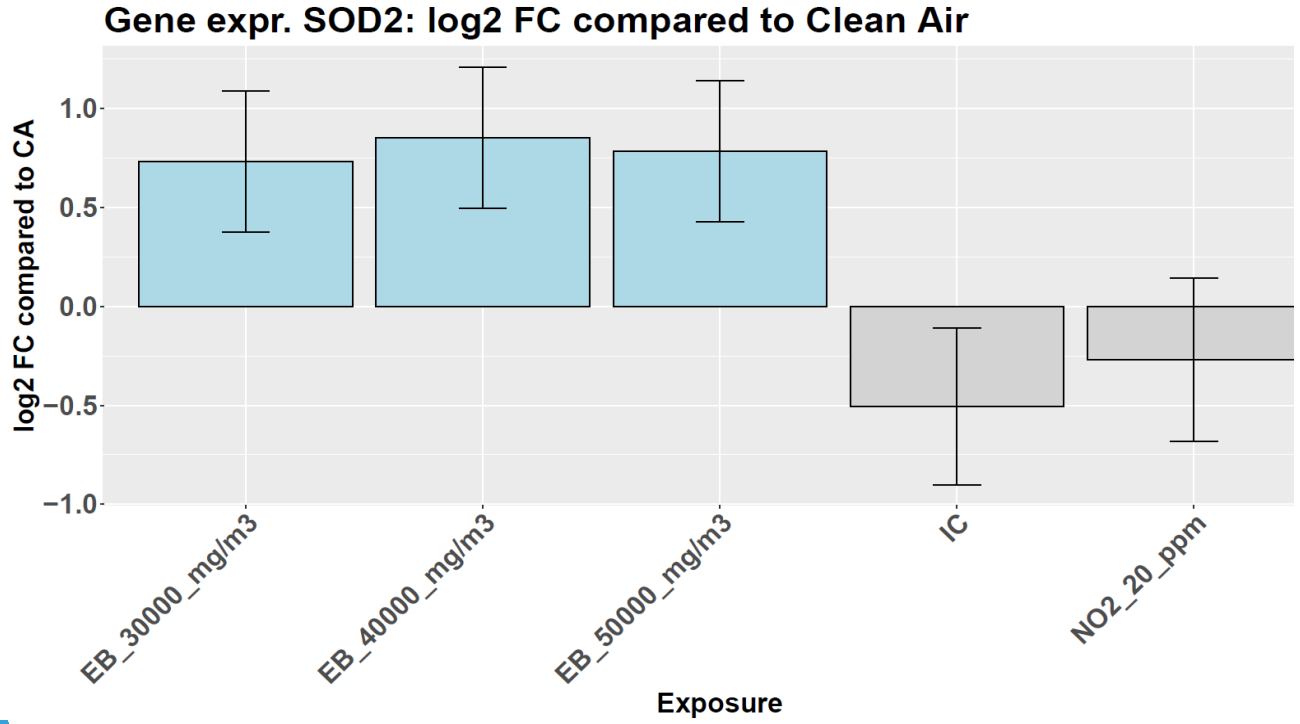


Same trend for *CCL2* and *IL6*

INFLAMMATION (PROTEIN SECRETION)



OXIDATIVE STRESS (GENE EXPRESSION)



HMOX1 not a relevant marker for EB exposure

CONCLUSIONS

- Successful optimization of ALI exposure system for a single substance EB
- Experimental conditions optimized to achieve a deposition efficiency that resulted in dose-related biological changes
- The data demonstrated consistency in effect levels when comparing cell viability in the ALI experiments with known *in vivo* effects.
- Publication ready for submission
- Other cases: gasoline (SOT ePoster #1177) & naphthalene

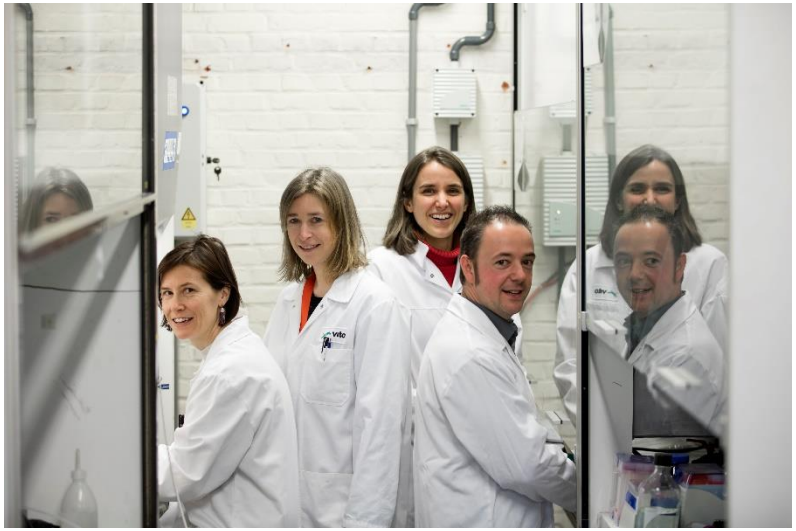
ACKNOWLEDGEMENTS

PETA INTERNATIONAL SCIENCE CONSORTIUM LTD.

- Monita Sharma
- Amy J. Clippinger

ExxonMobil

- Lize Deferme
- Katy O Goyak
- Marusia A Popovech



Evelien Frijns (aerosol expert)
evelien.frijns@vito.be
Sandra Verstraelen (biomolecular expert)
sandra.verstraelen@vito.be

Researchers: Frederick Maes, Lieve Geerts, Sylvie Remy, Hilda Witters, Christine Vanhoof
Technicians: An Jacobs, Karen Hollanders, Jo Van Laer, David Poelmans, Rob Brabers, Masha Van Deun, Diane Bertels, Wilfried Brusten
Business developer: Sven Vercauteren