INSPiRE PROJECT: *IN VITRO* INHALATION TESTING OF SILANE & SURFACTANT COMPOUNDS

Case studies

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AGENDA

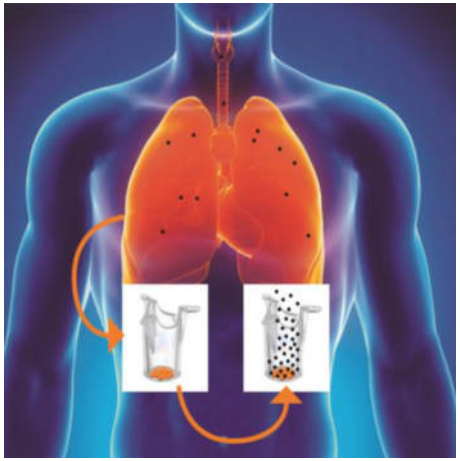
- VITO
- State of the art
- Air-liquid interface cell cultures & exposure techniques @VITO
- Generation & exposure infrastructure @VITO
- **Case study INSPiRE**

Test chemicals & controls	Generation setups	Exposure setups	Vapor/aerosol characterization – generated concentration	Delivered dose	VITROCELL® 6/4 exposure system & parameters
Test systems, parameters & endpoints	Experimental designs	Results	Troubleshooting	Next steps	Acknowledgements

VITO – FLEMISH INSTITUTE FOR TECHNOLOGICAL RESEARCH

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

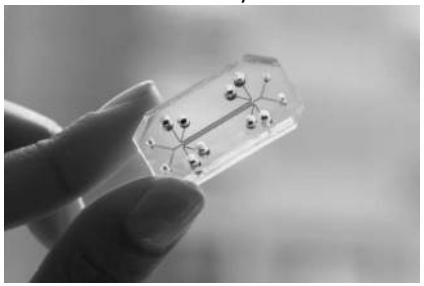
In vitro platform for inhalation testing

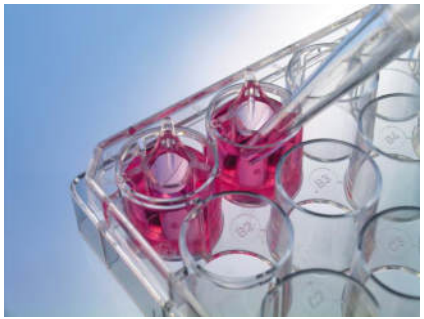


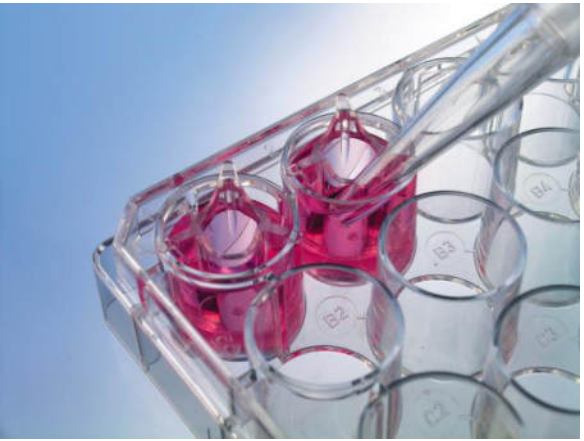
STATE OF THE ART

Non-guideline methods

human-relevant/advanced

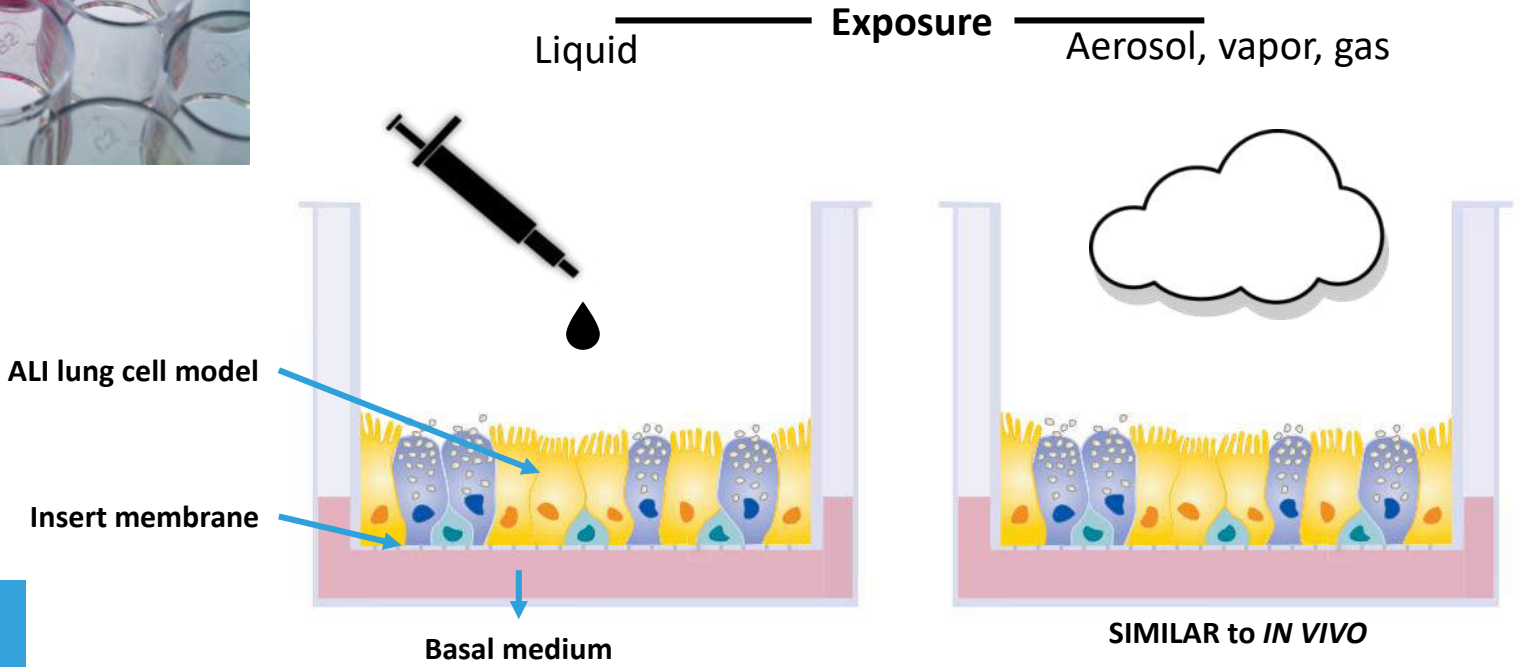






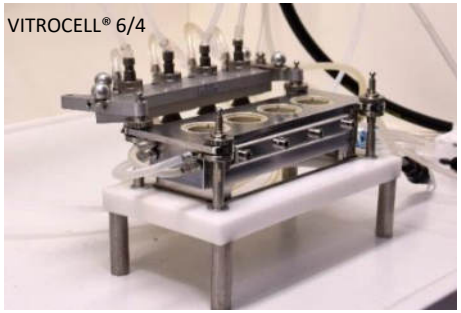
Air-liquid interface (ALI) expertise:

- Monoculture cell lines (BEAS-2B, Calu-3, A549)
- 3D tri-culture alveolar model (Luxembourg Institute of Science and Technology)
- 3D tissues from human donors (nasal or bronchial MucilAir™, Epithelix Sàrl, Swiss)



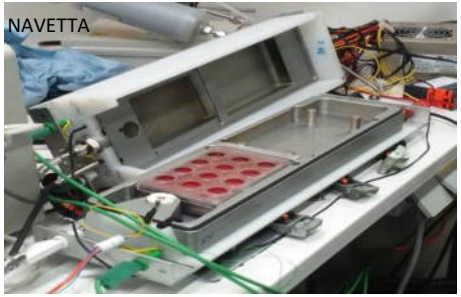
GENERATION AND EXPOSURE INFRASTRUCTURE @VITO

VITROCELL® 6/4



VITROCELL® 24/48

NAVETTA



VITROCELL® Cloud 12

Wet generation

- Vibrating mesh nebulizer
- Atomizers

Dry generation

- PreciseInhale®
- Rotating Brush Generator

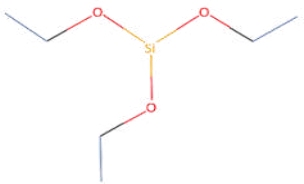
INSPiRE: *IN vitro* System to Predict Respiratory toxicity

- Demonstrate usefulness of different *in vitro* test systems
- Predict ability of chemicals to cause portal-of entry effects on human respiratory tract

		Differences between project phases
Phase **1** (Completed)	Assess the respiratory toxicity of **triethoxysilane** in **BEAS-2B** cells	https://www.piscltd.org.uk/inhalation-webinars/
Phase **2** (Ongoing)	Assess the respiratory toxicity of **silanes and surfactants** in **BEAS-2B** cells	Key differences between Phase 1 and Phase 2: • Reduce exposure time to **30 minutes** • **Additional test substances** • Adding **'true' negative control** • Using **nitrogen** gas as a carrier control for silanes • Testing **only 4 cytokines** • **ALI post-exposure** • **Removed bovine pituitary extract** from cell media
Phase **3** (Ongoing)	Assess the respiratory toxicity of **silanes and surfactants** in **MucilAir™**	Key differences between Phase 2 and Phase 3: • Using a **3D** model • Assessing **additional endpoints** • Adding **7 day recovery period**

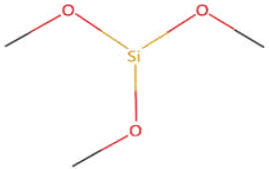
SILANES

- Acute toxicity by inhalation
- Hydrolyze quickly: ethanol, methanol, hydrochloric acid
- Corrosion protection, adhesion promotion, surface modifications



Triethoxysilane (TES)

GHS2, CAS 998-30-1
Boiling point: 134-135°C
Vapor pressure: 20.25 mmHg @20°C

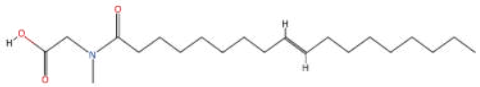


Trimethoxysilane (TMS)

GHS1, CAS 2478-90-3
Boiling point: 86°C
Vapor pressure: <7.2 mmHg @20°C

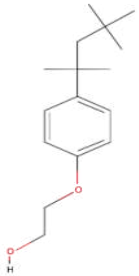
SURFACTANTS

- OS: Corrosion inhibitor in aerosol products
- TX-100: Lyse cells to extract protein/organelles or to permeabilize the living cell membrane for transfection



Oleoyl sarcosine (OS)

GHS4, anionic, CAS 110-25-8
Boiling point: 1413°C
Vapor pressure: no data



Triton X-100 (TX-100)

Non-ionic, CAS 9002-93-1
Boiling point: > 200°C @1.013 hPa
Vapor pressure: < 1.00 mmHg @20 °C

NEGATIVE

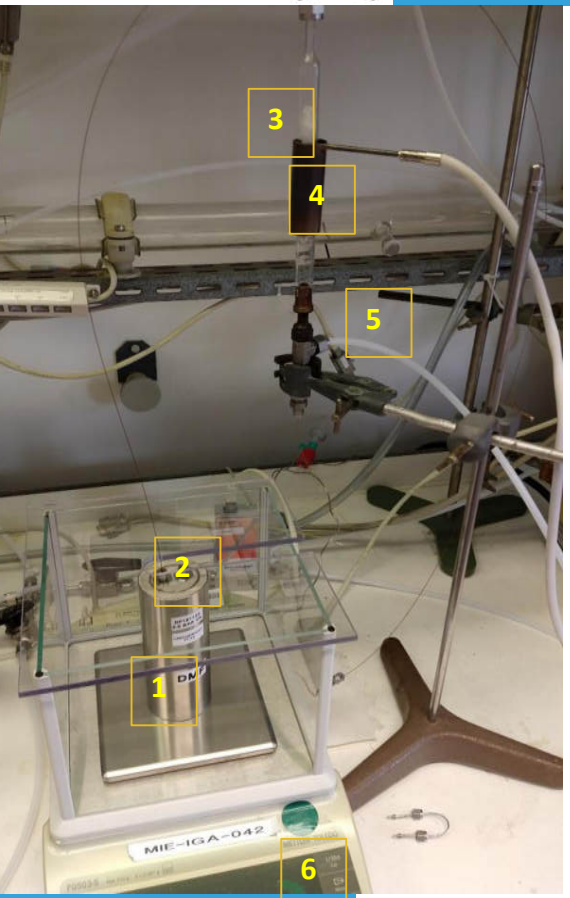
- Clean air (CA) – Compressor, HEPA/Active Carbon filter
- Nitrogen (N_2) – Storage tank
- Sodium Chloride (NaCl) – Atomization 0.9%

POSITIVE

- Nitrogen dioxide (NO_2) – Gas cylinder, N_2 dilution

- BEAS-2B: 25 ppm
- MucilAir™: 800 ppm

GENERATION SETUP SILANES

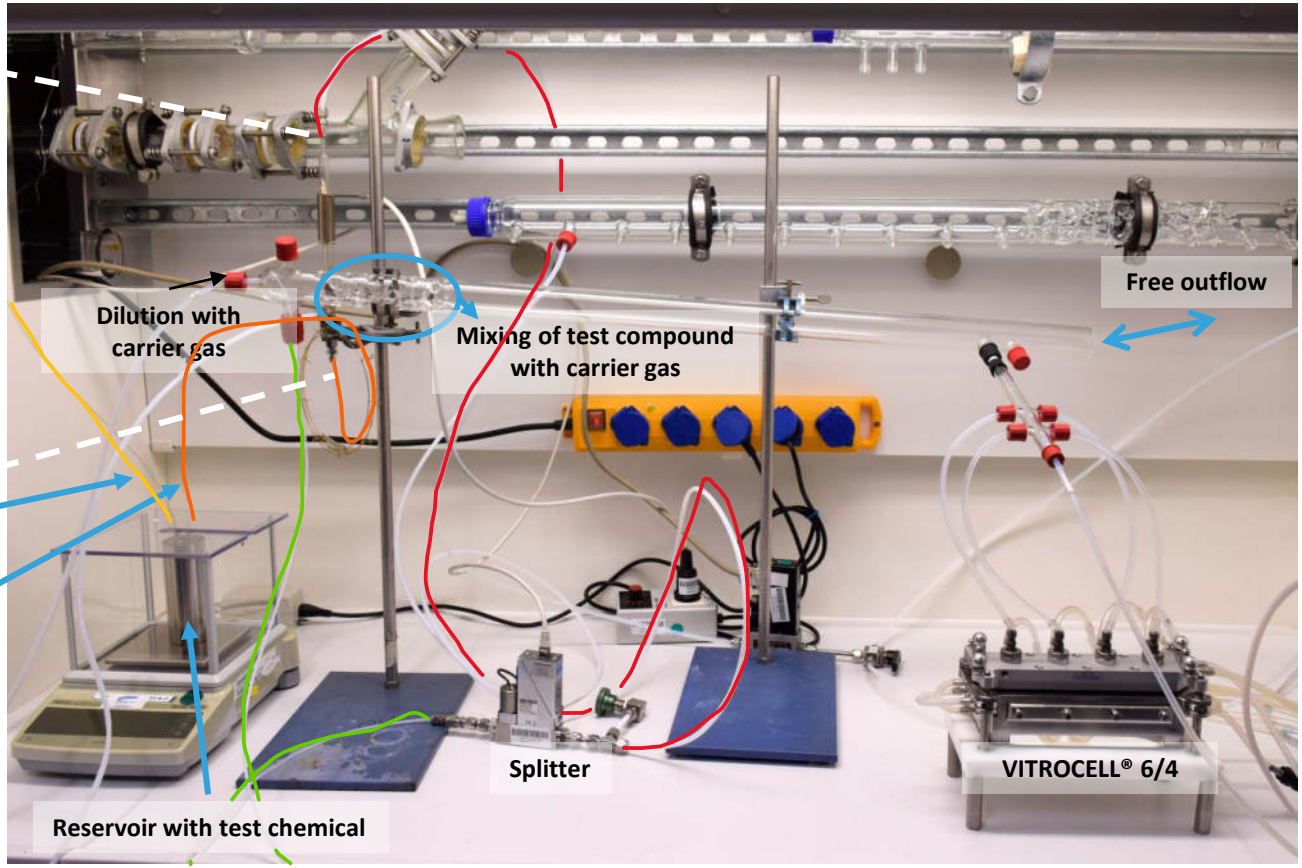


Capillary dosage (Goelen *et al.* 1992*)

1. Recipient with silane on analytical balance
2. Pressure on closed recipient
3. Outgoing liquid retained by cotton plug
4. Liquid evaporated by local heating element
5. Silane vapor transferred by dry N_2 flow to glass distribution line
6. Weight loss monitored

*Goelen E, Lambrechts M, Geyskens F, Rymen T (1992). Development and Performance Characteristics of a Capillary Dosage Unit with in Situ Weight Sensor for the Preparation of Known Amounts of Gaseous Voc's in Air. International Journal of Environmental Analytical Chemistry, 47 (4): 217-225.

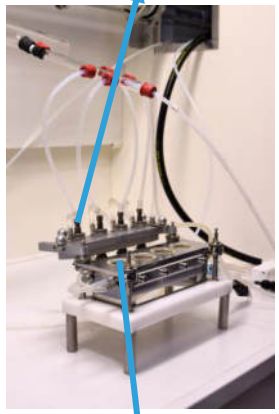
EXPOSURE SETUP SILANES



Pressure on the reservoir

Transport of test chemical out of reservoir

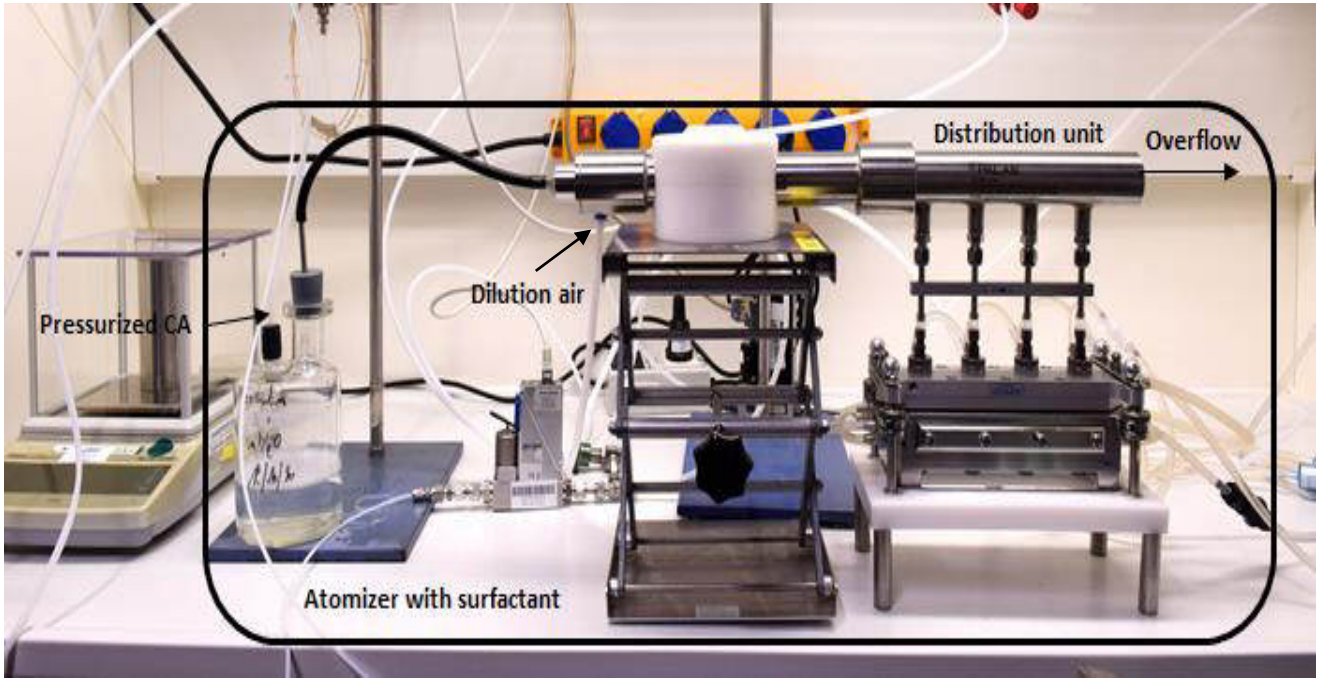
Inlet for vaporized test chemical



Position for inserts with cells

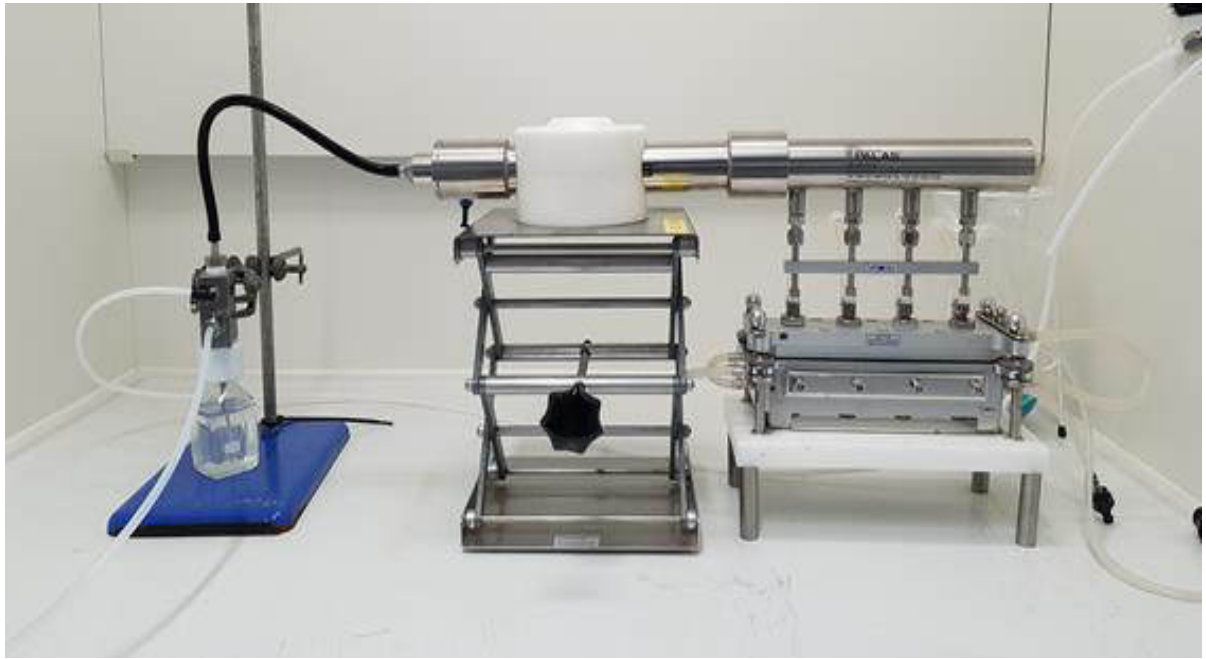
EXPOSURE SETUP TX-100

- TX-100 diluted with MilliQ
- Range <2.5% (BEAS-2B)
- Range <10% (MucilAir™)

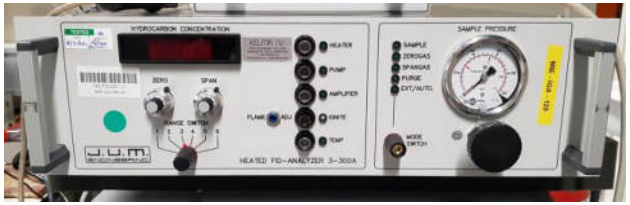


EXPOSURE SETUP OS

- OS with 10% ethanol to reduce viscosity
- 2.5 bar
- Flow dilution 0, 2, and 5 lpm
- 188-647 mg/m^3 (MucilAir™)



VAPOR/AEROSOL CHARACTERIZATION – GENERATED CONCENTRATION



Silanes

- Stability of silanes in carrier gas was online monitored with a FID analyzer (JUM model 3-300) and generated concentration was calculated.
- Dose: Insert membrane with cells removed with scalpel blade and stored in a 15 ml tube at -20°C. ICP-AES analysis (to be performed).

OS

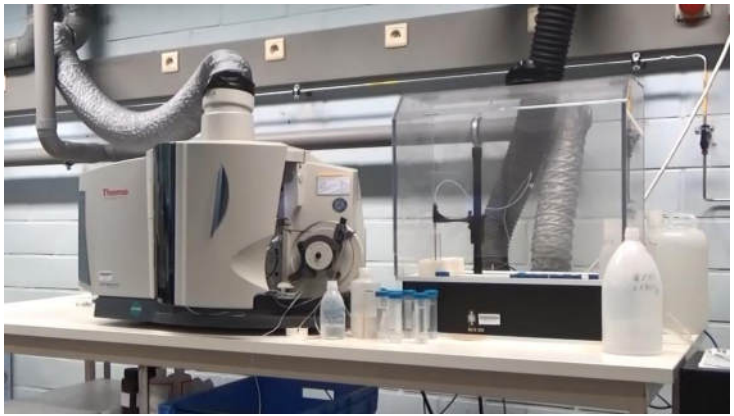
- Generated mass concentration determined by sampling on a 25mm quartz fiber filter and weighing the filter before and after sampling. Together with the sampling volume, mass concentration was calculated (mg/m^3).
- Dose: Taped dry inserts, after OS exposure, rinsing membrane with 100 µl ethanol. Ethanol was collected for LC-MS analysis.

TX-100

- Dose: Insert membrane with cells removed with scalpel blade and stored in a 15 ml tube at -20°C. LC-MS analysis (to be performed).

SILANES

- ICP-AES (inductively coupled plasma-atomic emission spectroscopy)
- Silicium standard in 5% TMAH (tetramethylammoniumhydroxide); digestion in TMAH
- Insert membrane with cells

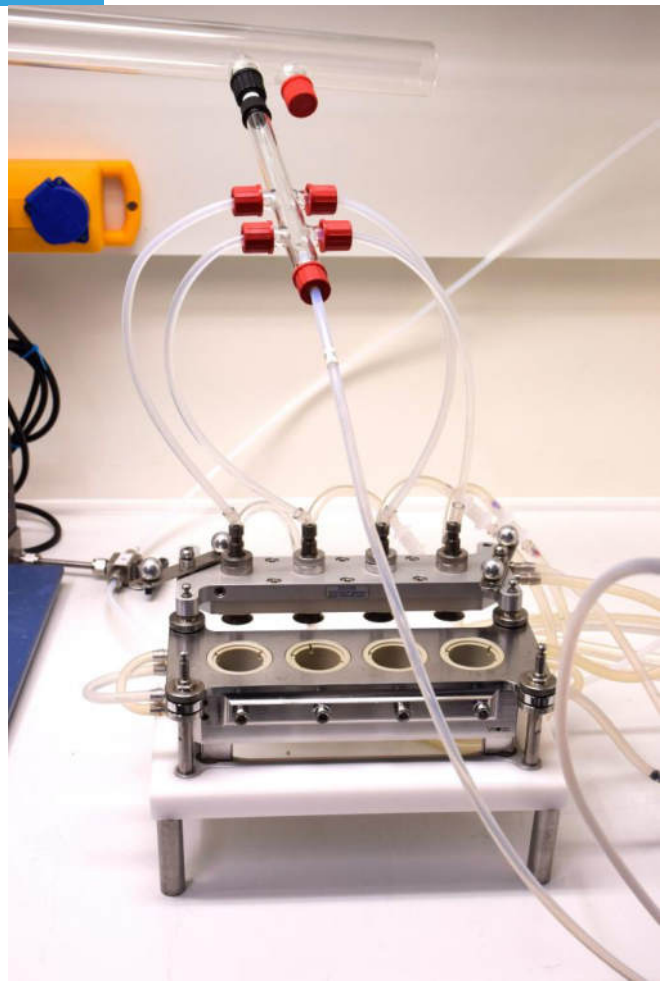


SURFACTANTS

- LC-MS (liquid chromatography-mass spectrometry)
- Mobile phase A: 60% water + 0.02% formic acid; mobile phase B: 40% methanol
- Taped inserts, rinsing with ethanol
- Insert membrane with cells

VITROCELL® 6/4 EXPOSURE SYSTEM & PARAMETERS

Parameters	
Flow rate over cells	3 milliliter per minute (mlpm)
Flow rate elsewhere	Depends on concentration/dose needed
Trumpet height	3 mm
Conditioning	• Temperature during exposure: 37 °C • Dry exposure because of reactivity silanes



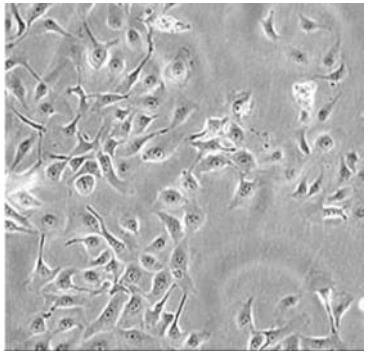
In vivo Key Events silanes
Cell death
Loss of epithelial barrier
Secretion of inflammatory cytokines
Pulmonary edema/hemorrhage

In vivo Key Events surfactants
Interaction with pulmonary surfactant
Disruption epithelial lining & cell membranes
Denaturation proteins
General cytotoxicity

BEAS-2B: 2D normal human bronchial epithelial cell line

Endpoints:

- Cell viability (cell metabolization activity; PrestoBlue™)
- Cytotoxicity (cell membrane integrity, release of lactate dehydrogenase (LDH); CytoTox-ONE™)
- Inflammatory markers (IL-2, IL-6, IL-8, TNF-α; V-PLEX, Meso Scale Discovery)



Parameters	
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	50.000 cells/cm²
Growth protocol	48 h submerged growth, exchange bronchial epithelial growth medium (BEGM without BPE) for bronchial epithelial basal medium (BEBM) at the day of ALI exposure

Pseudostratified epithelium

MucilAir™: 3D human epithelial tissue model

-> normal bronchial male & female mono-donor tissues

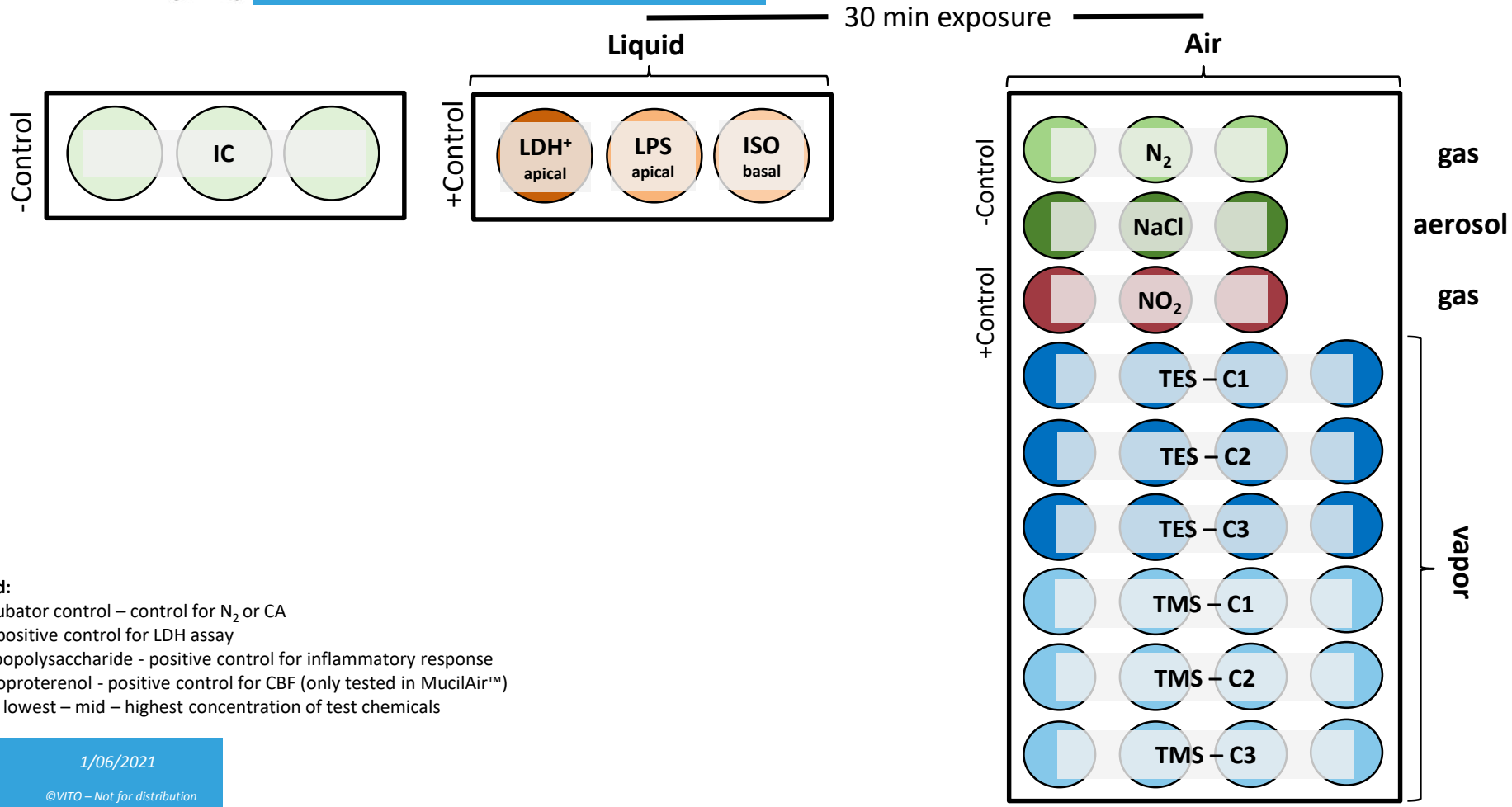
Endpoints:

- Cell viability
- Cytotoxicity (LDH Assay Kit-WST)
- Inflammatory markers (IL-2, IL-6, IL-8, TNF-α)
- Barrier integrity (Transepithelial electrical resistance (TEER); Millicell ERS-2)
- Cilia beating frequency (CBF; SAVA system)
- Morphology (hematoxylin and eosin (H&E) staining)

Cerba Research

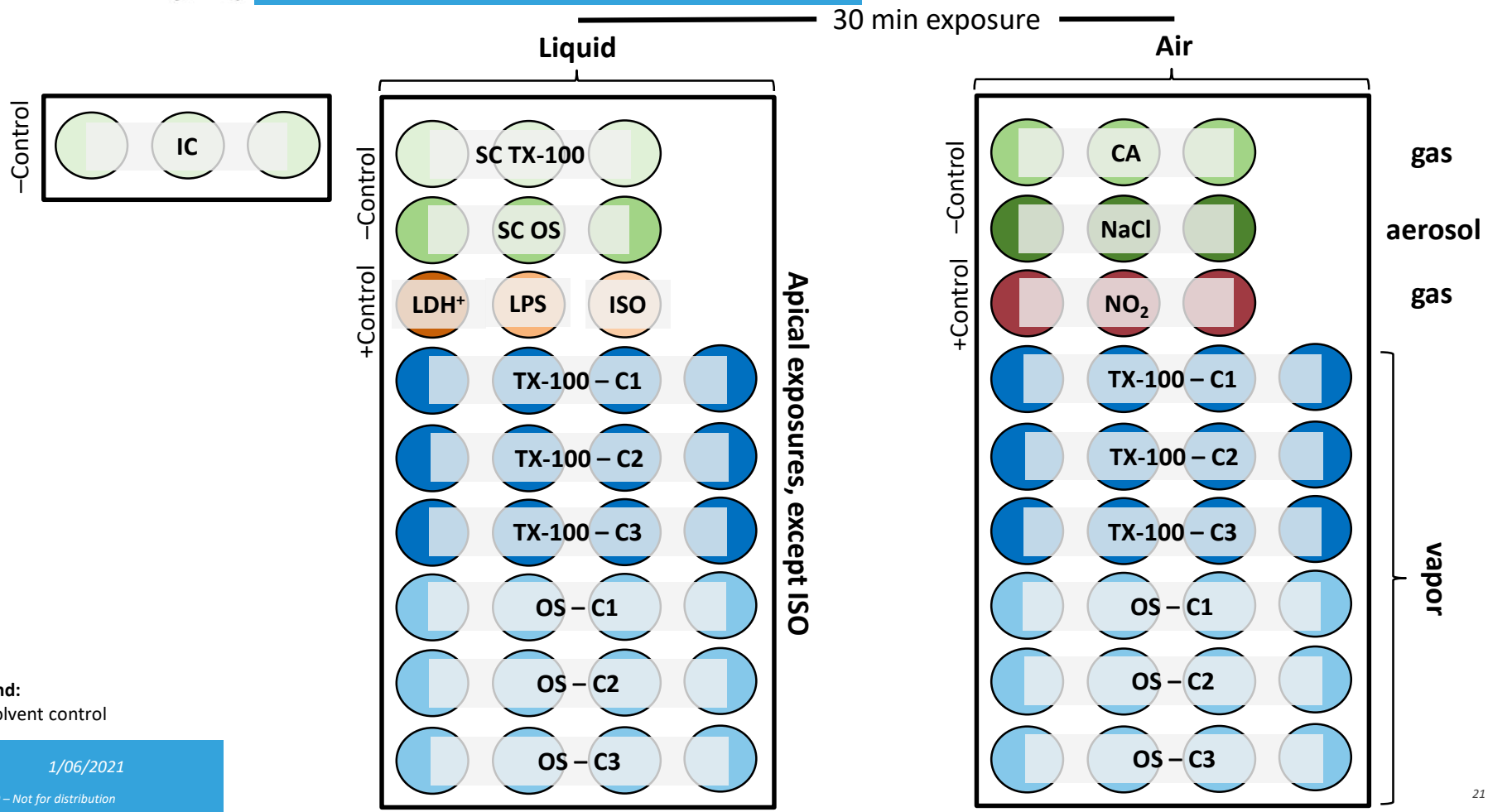
Parameters	
Type of inserts	Corning® Transwell® polyester membrane inserts (Sigma-Aldrich), 24-well
Thickness epithelium	40-50 µm
Protocol	• Commercially available from Epithelix Sàrl (Swiss) -> standardized platform & maintenance protocol • 24 h before exposure: apical wash of each insert to remove mucus + basal TEER measurement

EXPERIMENTAL DESIGN SILANES



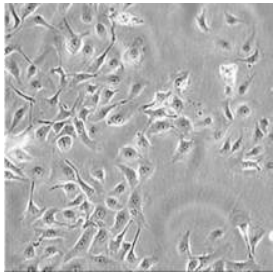
Legend:
IC: incubator control – control for N_2 or CA
LDH^+: positive control for LDH assay
LPS: lipopolysaccharide - positive control for inflammatory response
ISO: isoproterenol - positive control for CBF (only tested in MucilAir™)
C1-C3: lowest – mid – highest concentration of test chemicals

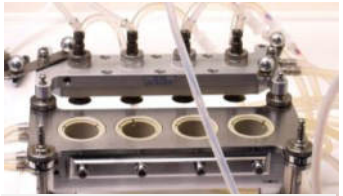
EXPERIMENTAL DESIGN SURFACTANTS



Legend:
SC: solvent control

EXPERIMENTAL DESIGN BEAS-2B





At least 4 replicates/condition

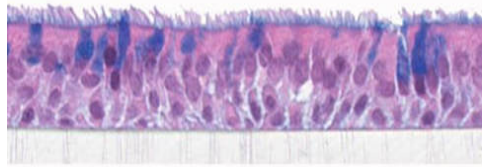
At least 3 independent biological experiments

30 min exposure

ALI post-exposure (PE)	**ICP-AES/ LC-MS** cells+basal	**LDH** basal	**PrestoBlue** cells	**Inflammatory markers** basal
No	**1**			
30 min		**3**		
19-24 h		**3**	**3**	**3**

For each experiment of silanes: 10 conditions * 13 readouts -> **130 readouts**
For each experiment of surfactants: 17 conditions * 13 readouts -> **221 readouts**

EXPERIMENTAL DESIGN MUCILAIR™



At least 4 replicates/condition

At least 3 independent biological experiments

30 min exposure

ALI post-exposure (PE)	**ICP-AES/ LC-MS** Cells+basal	**LDH** basal	**PrestoBlue** cells	**Inflammatory markers** basal	**TEER** /	**CBF** /	**H&E** cells
No	**1**						
19-24 h		**3**	**1**	**3**	**3**	**3**	**1**
7 d*		**2**	**2**	**2**	**2**	**2**	**1**

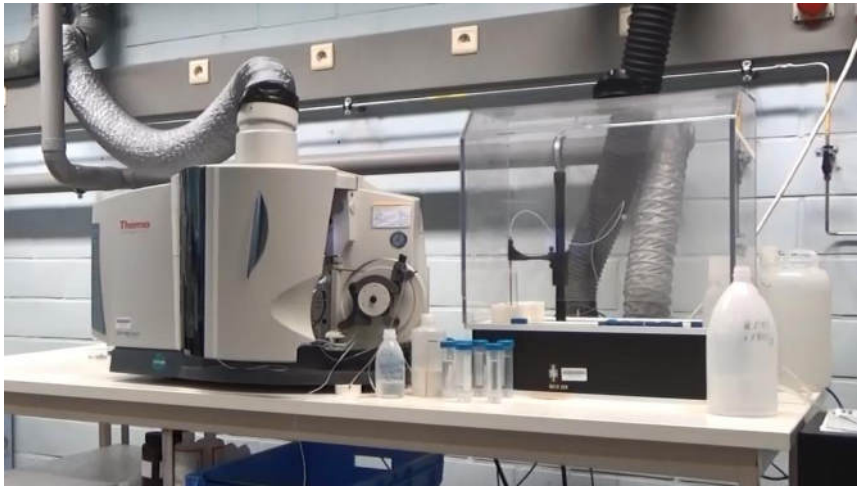
**Medium refreshment on d 3 & 6*

For each experiment of silanes: 10 conditions * 26 readouts -> **260 readouts**
For each experiment of surfactants: 17 conditions * 26 readouts -> **442 readouts**

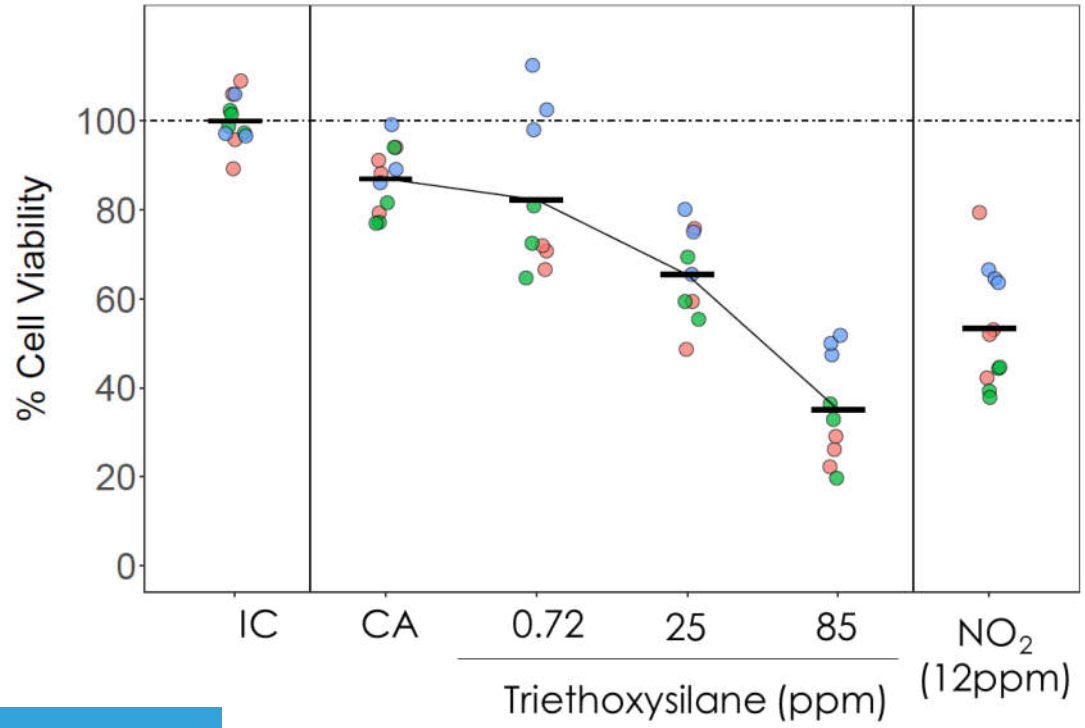
PHASE I RESULTS (COMPLETE)

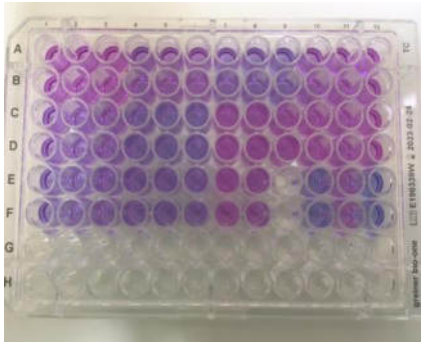
GENERATED CONCENTRATION *VERSUS* DELIVERED DOSE (ICP-AES) OF TES

Generated concentration		Delivered dose in cells (µg)			
ppm	**mg/m³**	N=1	N=2	N=3	**Mean +/- SD**
0.72	**4.9**	<1.2	<1.2	<1.2	**<1.2**
25	**169.8**	2.6	3.3	3.8	**3.2 +/- 0.6**
85	**577.2**	15.4	17.4	20.8	**17.9 +/- 2.7**
		Delivery efficiency: 14%			

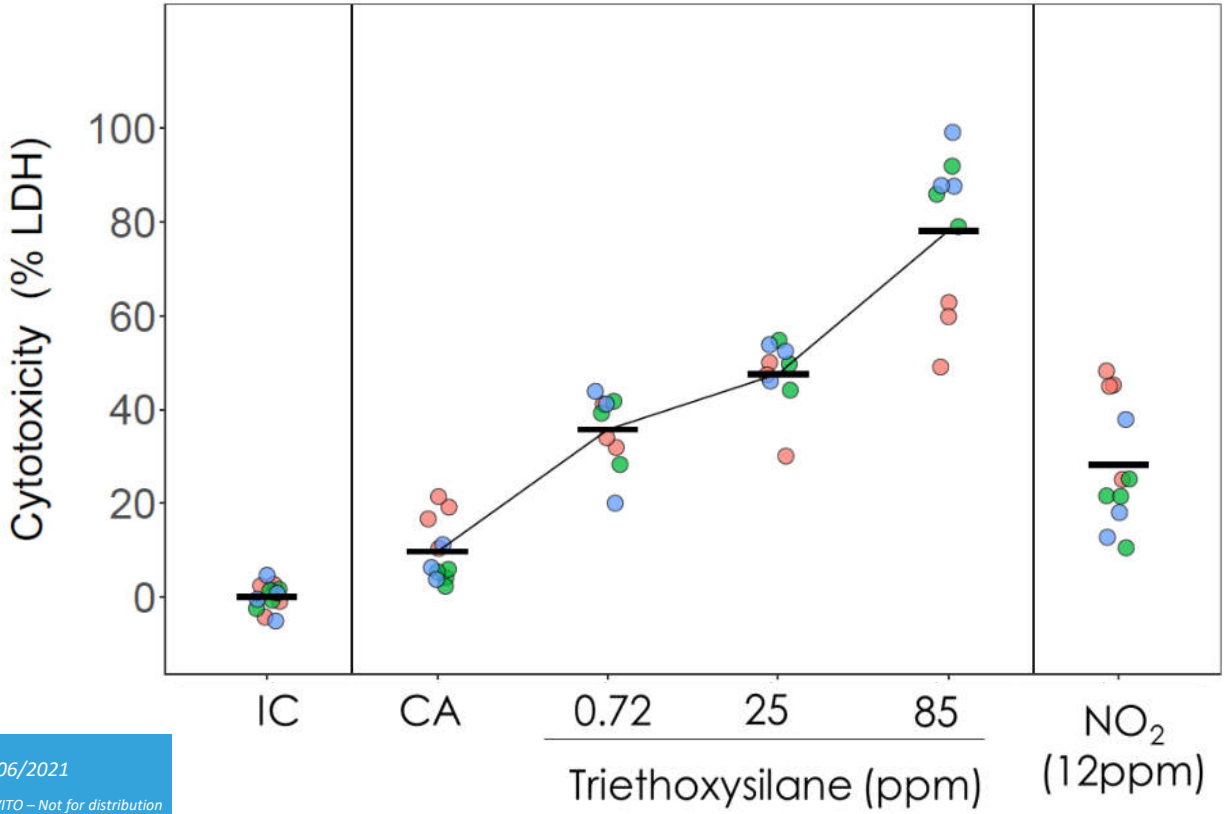


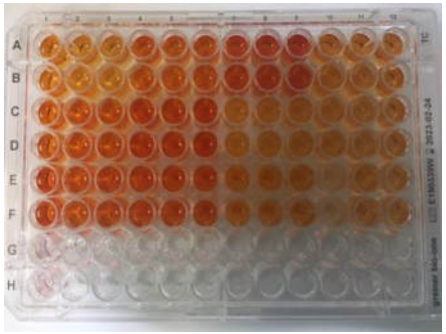
CELL VIABILITY (PRESTOBLUE™, 19-24 H PE)



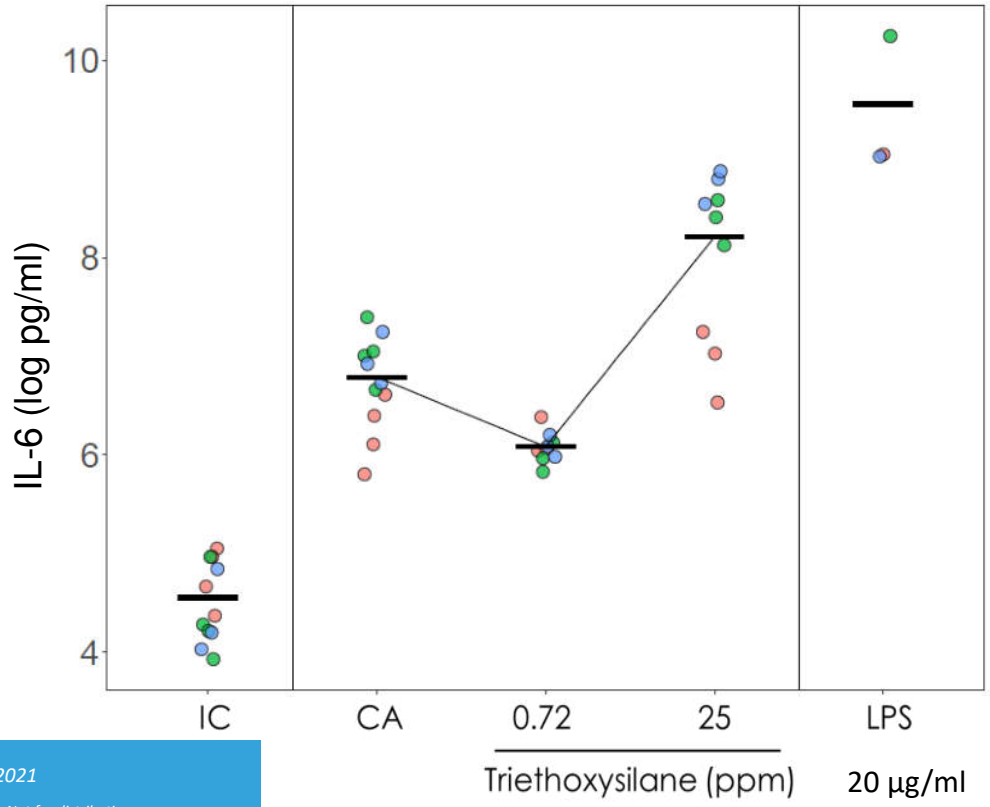


CYTOTOXICITY (LDH RELEASE, 30 MIN PE)





INFLAMMATION (CYTOKINE RELEASE, 19-24 H PE)



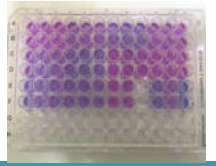
Same trend for:
IL-2, IL-4, IL-8, IL-10, IL-12p70, IL-13, TNF-α

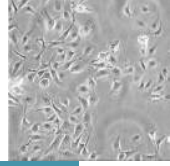
Below detection: IL-1β and IFN- ɣ

PHASE 2 & PHASE 3 (ONGOING)

		Differences between project phases
Phase **1** (Completed)	Assess the respiratory toxicity of **triethoxysilane** in **BEAS-2B** cells	https://www.piscltd.org.uk/inhalation-webinars/
Phase **2** (Ongoing)	Assess the respiratory toxicity of **silanes and surfactants** in **BEAS-2B** cells	Key differences between Phase 1 and Phase 2: • Reduce exposure time to **30 minutes** • **Additional test substances** • Adding **'true' negative control** • Using **nitrogen** gas as a carrier control for silanes • Testing **only 4 cytokines** • **ALI post-exposure** • **Removed bovine pituitary extract** from cell media
Phase **3** (Ongoing)	Assess the respiratory toxicity of **silanes and surfactants** in **MucilAir™**	Key differences between Phase 2 and Phase 3: • Using a **3D** model • Assessing **additional endpoints** • Adding **7 day recovery period**

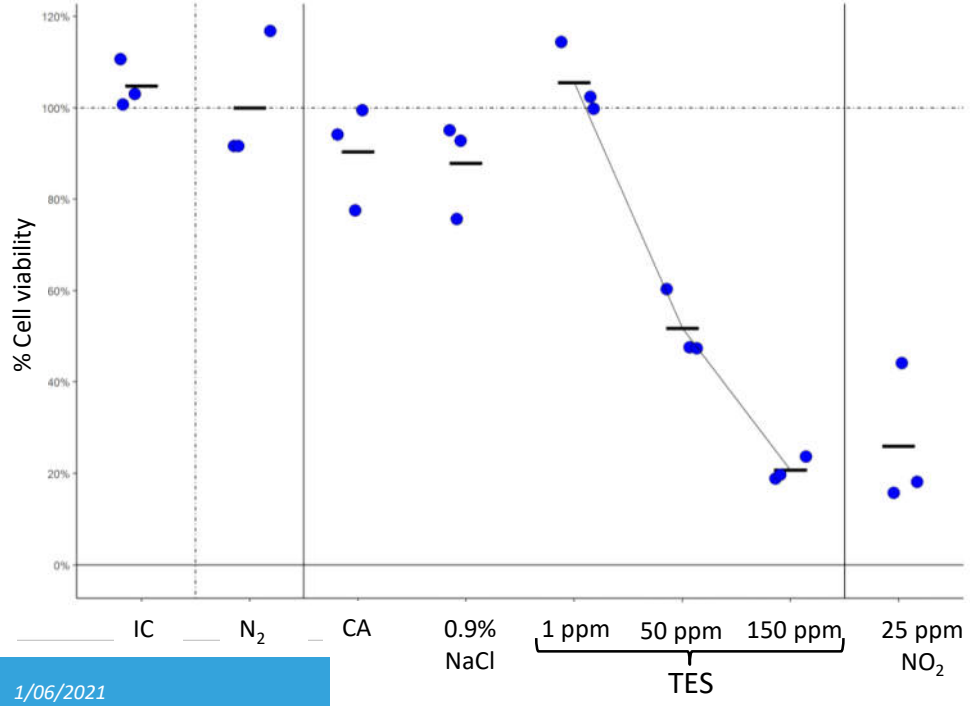
PHASE II RESULTS (ONGOING)



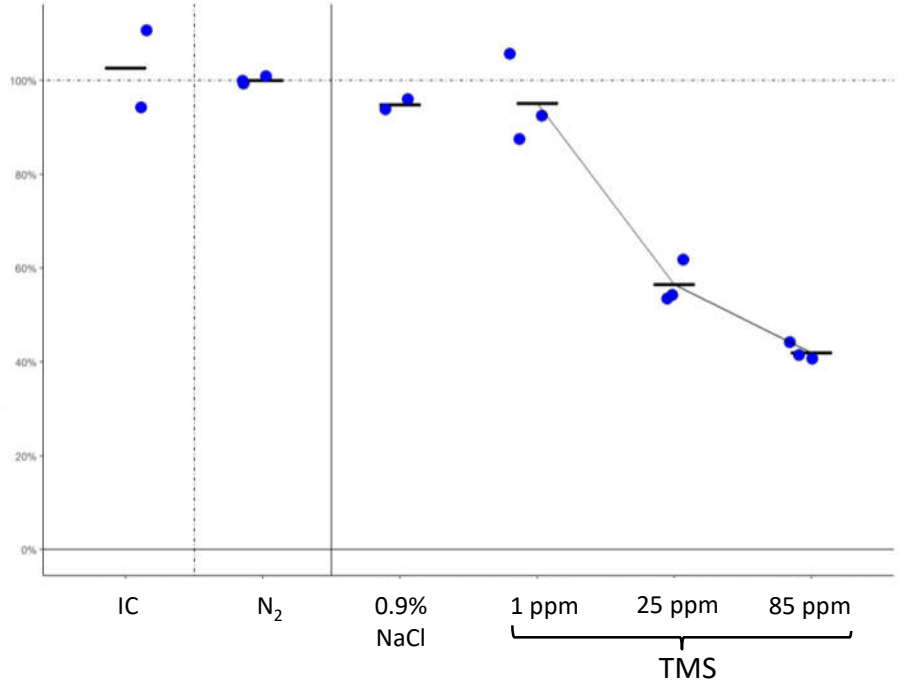


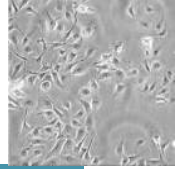
CELL VIABILITY (PRESTOBLUE™, 19-24 H PE)

TES (vapor)



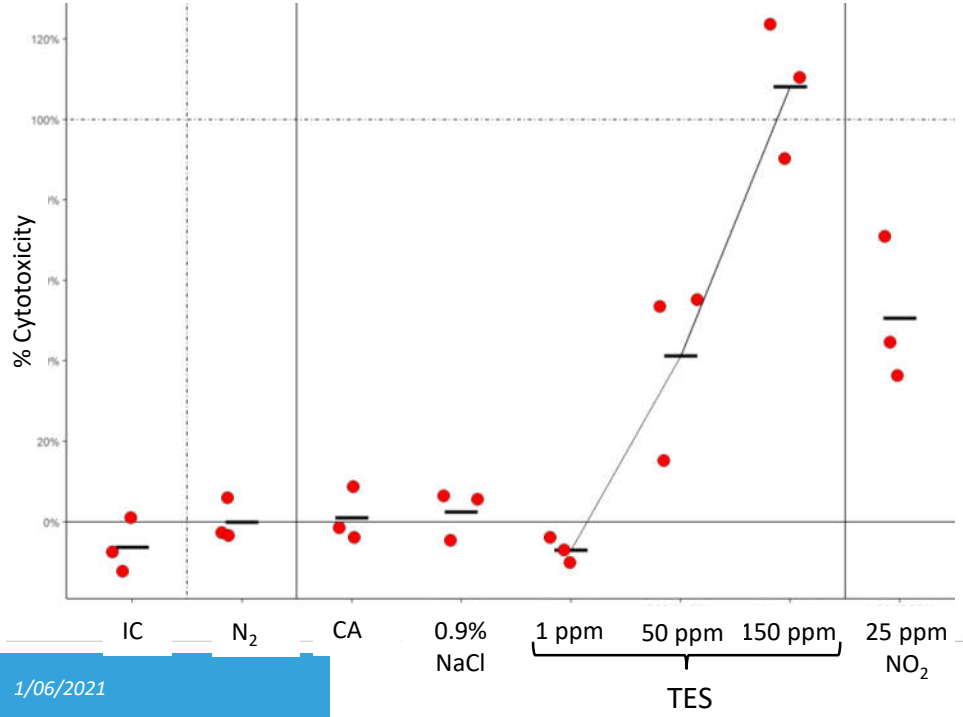
TMS (vapor)



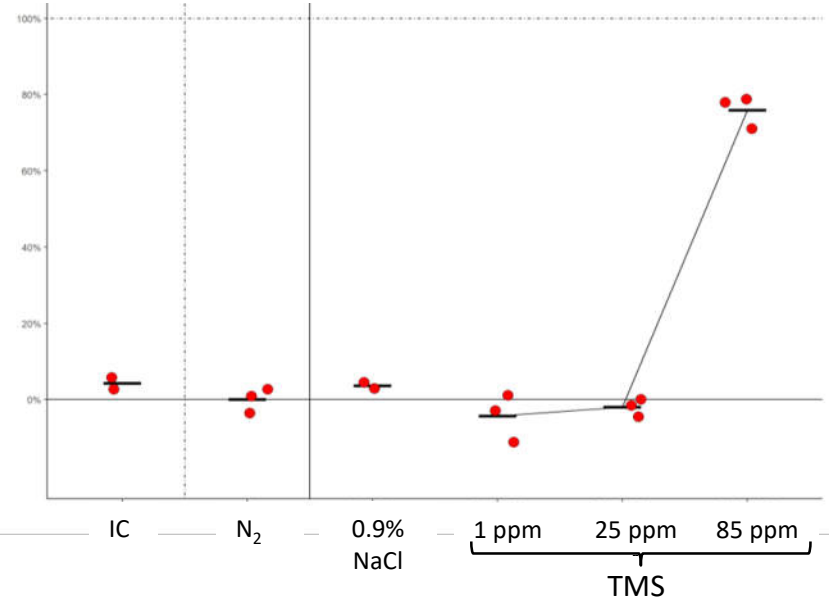


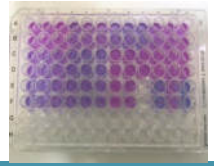
CYTOTOXICITY (LDH, 19-24H PE)

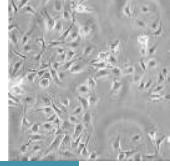
TES (vapor)



TMS (vapor)

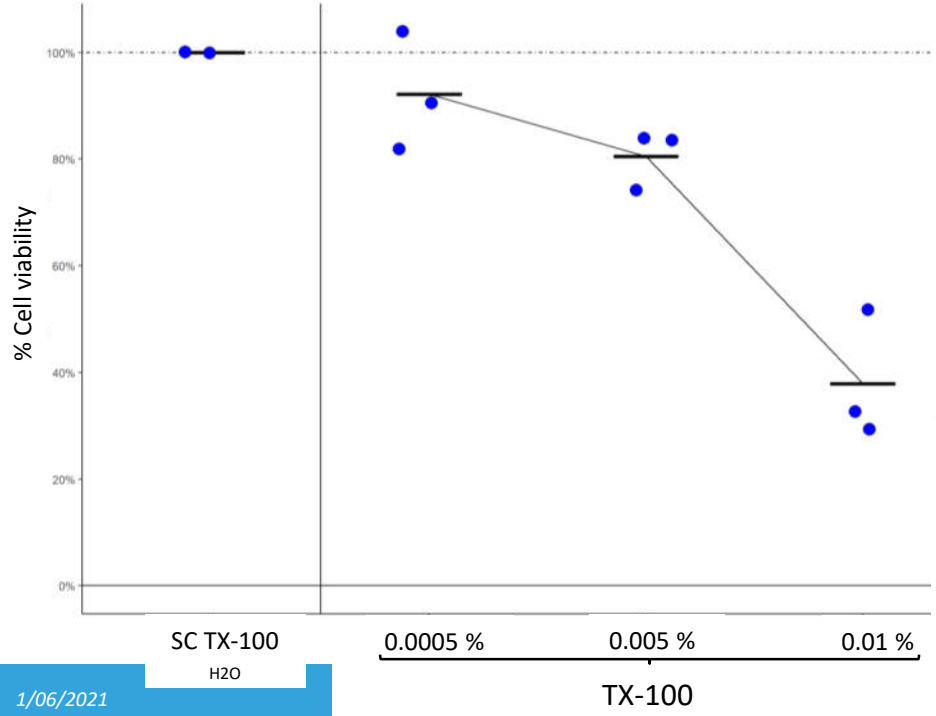




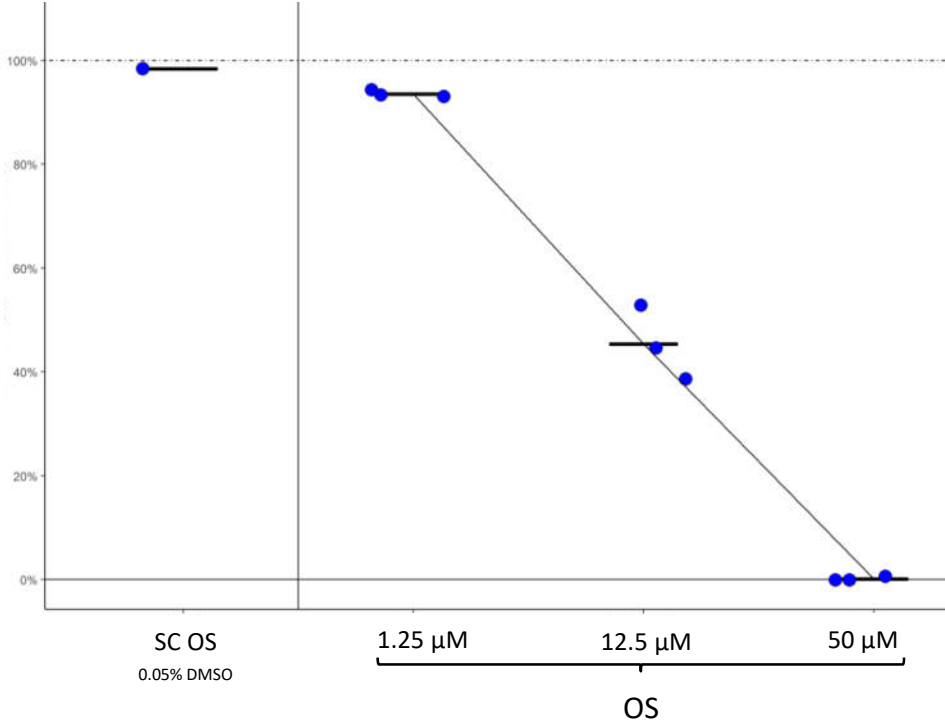


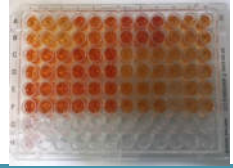
CELL VIABILITY (PRESTOBLUE™, 19-24 H PE)

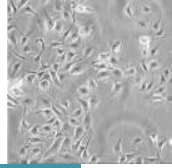
TX-100 (liquid)



OS (liquid)

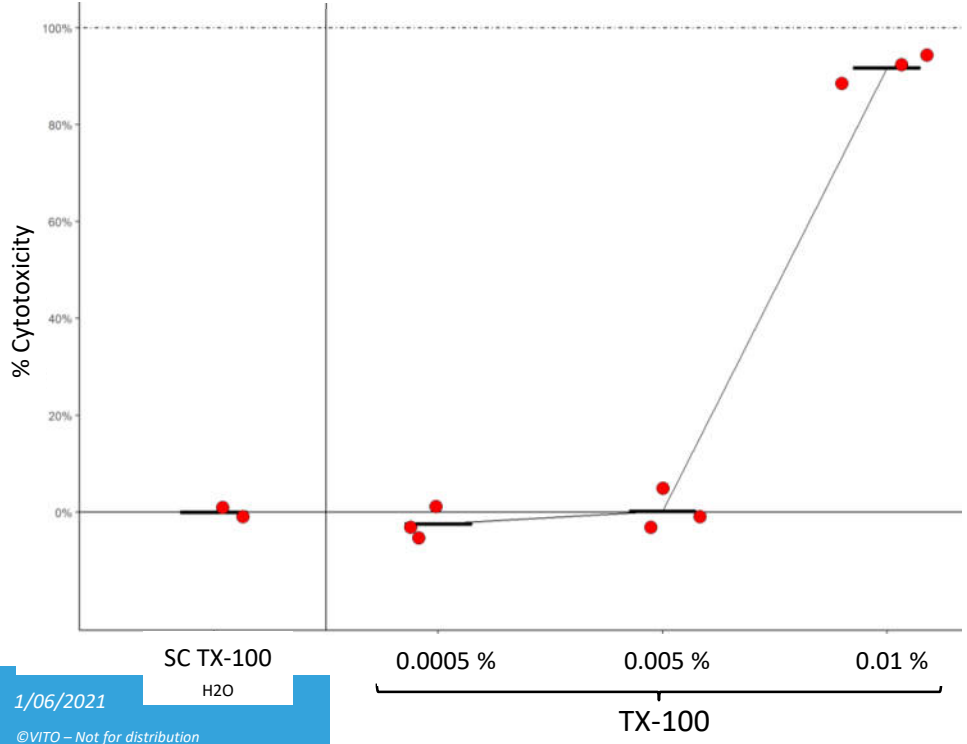




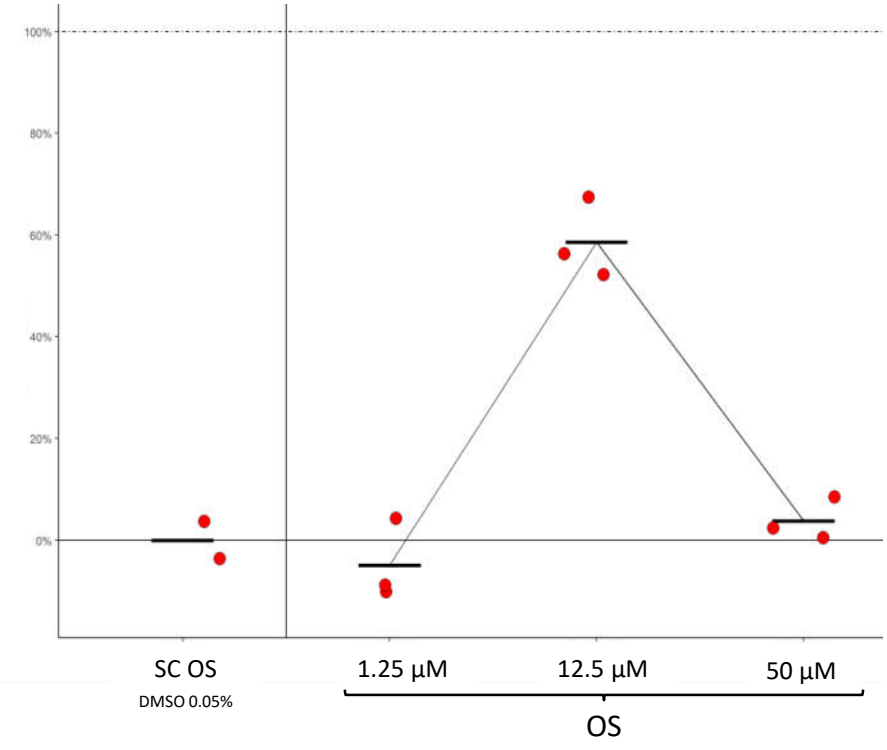


CYTOTOXICITY (LDH, 19-24H PE)

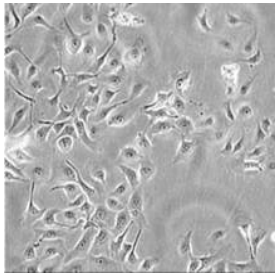
TX-100 (liquid)



OS (liquid)



TROUBLESHOOTING 2D BEAS-2B

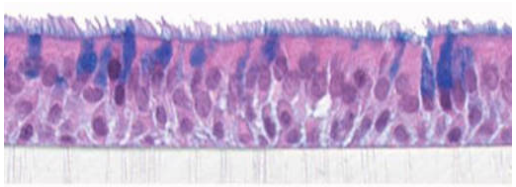


LDH read-out 30 min PE (CytoTox-ONE™, fluorescence-based)

- Lot of handlings at day of exposure -> difficult to perform LDH read-out 30 min PE
- Kept the samples at -20°C or 4°C for LDH read-out the next day -> does not work!
- LDH read-out same day of exposure (extra technician) or switch to absorbance-based kit

Exclusion 1 run because trumpets were adjusted with wrong insert (Greiner instead of Sigma, ≠ 0.5-1 mm)

TROUBLESHOOTING 3D MUCILAIR™



Some variability observed:

- Read-out NO_2 positive control
  - 800 ppm very high!
  - Responder and non-responders?
  - Suggestions for good positive controls (aerosol, gas, vapor)?

- Quantity and quality of mucus?

NEXT STEPS

- Fix Troubleshooting
- Obtain at least 3 valid independent runs in BEAS-2B & MucilAir™ for silanes and surfactants
- Determine if the test systems can detect the decrease in toxicity that correlates with increasing carbon length, which is not evident from available animal inhalation toxicity data
- Compare liquid exposure method and aerosol exposure method for surfactants
- Determine advantages of using a 2D cell line (BEAS-2B) versus a 3D human reconstructed tissue model (MucilAir™)
- *In vitro* to *in vivo* translation (IVIVE)

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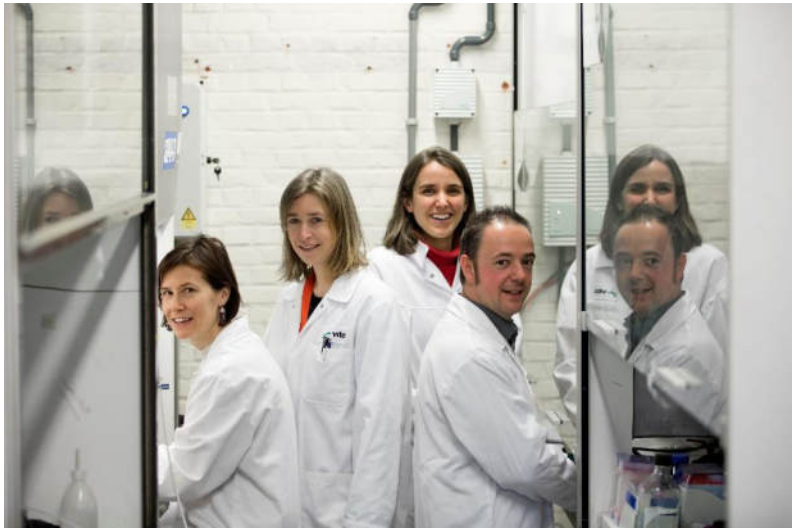
Links with:
- Engineering department
- Chemical testing department
- Biomarker discovery team



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