ROLE FOR IN VITRO MODELS IN THE TOXICOLOGICAL EVALUATION OF COMPLEX MIXTURES, SUCH AS EMISSIONS OF COMBUSTION PROCESSES

INGEBORG M. KOOTER
Introduction of new emission standards (PM mass) lowers the engine emissions from several transport modes. Implementation of new engine/emission control technologies and renewable fuels to meet the standards can lead to changes in the chemical mixture of pollutants. So, while regulatory requirements are met, the actual health effects of these reductions often are untested.
The toxicity of combustion of biodiesel at lower PM emission vs regular diesel

**PM mass**

1. PM mass (g kWh⁻¹) emissions for the fuel types and blends tested (6 ETC, except B10, B5 ETC and PPO 4 ETC). Error bars indicate the standard error of the mean. *, **, *** significantly different from B0 at P < 0.05, < 0.01, < 0.001 respectively.

**PM cytotoxicity**

*Fig. 5.* Relative cytotoxicity for the fuel types and blends tested, in % compared to positive control, 1% Triton-X100. Each bar represents 3 ETC. Error bars indicate the standard error of the mean. *, **, *** significantly different from the mean of B0 at P < 0.05, < 0.01, < 0.001 respectively.

Atm. Env. 2011

Kooter et al.
Atm. Env. 2011
Stronger biological responses at lower mass concentrations of nvPM from aircraft turbine engines

PM mass

PM inflammatory response (IL-8)

Table 2 Physical properties of non-volatile exhaust particles

<table>
<thead>
<tr>
<th>Fuels and thrust levels</th>
<th>Non-volatile particles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mass conc. (µg m⁻³) (SD)</td>
</tr>
<tr>
<td>Jet A-1</td>
<td></td>
</tr>
<tr>
<td>85% thrust</td>
<td>485 (14)</td>
</tr>
<tr>
<td>Ground-idle</td>
<td>4.6 (0.7)</td>
</tr>
<tr>
<td>HEFA blend</td>
<td></td>
</tr>
<tr>
<td>85% thrust</td>
<td>335 (8)</td>
</tr>
<tr>
<td>Ground-idle</td>
<td>1.3 (0.5)</td>
</tr>
</tbody>
</table>

CMD: count median diameter, HEFA: hydrotreated esters and fatty acid, SD: standard deviation, estM_{app}: estimated particle mass; cultures

*Estimated per hour exposure according to Jeantet et al.38

The introduction of new emission standards (PM mass) lowers the limits of engine emissions from several transport modes. Implementation of new engine/emission control technologies and renewable fuels to meet the standards can lead to changes in the chemical mixture of pollutants.

So, while regulatory requirements are met, the actual health effects of these reductions often are untested.

An intensive evaluation of the actual toxicity of emissions is absolutely needed, which could result in additional metrics to evaluate the health risks of pollutants.
Toxicity measurements linking PM and complex exhaust mixtures from engines operating under realistic conditions to health outcomes

for automotive, as well aircraft turbine, ship engines etc.

not only commonly used fuel, but also new fuels

Full chemical characterisation

regulated as well non-regulated compounds

Toxicological characterisation

Can in vitro models relevant for human exposure be the basis for such an evaluation to test for adverse health effects of source specific emissions?
RESPIRATORY MODELS TO STUDY INHALATION EFFECTS

The real situation is mimicked as much as possible
as complex as needed, as simple as possible
Epithelial cells cover airways and alveoli
Presently no easy to use testing system for inhalation route

Hiemstra et al. TIV 47 (2018)
Choice of cells

Cell lines

- Primary human lung epithelials cells
- Induced pluripotent stem cells

› Mono or co-cultured
VALIDITY IN VITRO SYSTEMS

1. Choice of cells

- Cell lines
- Primary human lung epithelial cells
- Induced pluripotent stem cells

Comparison study between A549, Beas-2B and primary epithelial cells (MucilAir) using CeO$_2$ nanoparticles air exposure:

- Mono or co-cultured

![Graphs showing IL-8 and LDH responses](image)

- Cell lines showed higher response for cytotoxicity and genotoxic dose

- MucilAir are less affected by air stream than cell lines
VALIDITY IN VITRO SYSTEMS

1. Choice of cells
- Cell lines
  - Human lung epithelials cells
  - Pluripotent stem cells
- Induced pluripotent stem cells

2. The cell culture system used
- Air-liquid interface (ALI), organ-on-a-chip models and microfluidics
- Organoids
- Precision cut lung slices

3. The type of exposure
- Addition of compounds to the medium of submerged cell cultures
- Air-exposure on ALI
- Gas or aerosol exposure on ALI

4. Its possibilities for valid read-outs to assess the effect of exposure
- Cell death, DNA breaks, barrier function, cytokine release, oxidative stress
- Omics techniques: protein and gene expression level
Cellular response of primary bronchial epithelial cells to diesel exhaust

- Helmond - TNO automotive PTC
- EuroV engine from city bus
- Braunschweig City Driving Cycle
- L/M/H: 34/82/206 \( \mu \text{g/m}^3 \)
- 1.9\% deposition
EFFECT OF THE EXPOSURE DURATION

6h post exposure

60 min  150 min  375 min

L/M/H: 0.14/0.43/1.29 mg/m³
EFFECT OF THE POST-EXPOSURE INCUBATION TIME

1h exposure

LDH

cytox

IL-8

protein

HMOX1
gene expr

L/M/H: 0.14/0.43/1.29 mg/m³
Gene expression vs protein expression of IL-8

1h exposure

L/M/H: 0.14/0.43/1.29 mg/m^3
EURO V DIESEL EMISSION – OXIDATIVE STRESS RESPONSE

L/M/H: 0.14/0.43/1.29 mg/m³

L/M/H: 34/82/206 µg/m³

HMOX1

NQO1

1 hrs exposure; 1:30/3/6h post exp

6 hrs exposure; 1:30h post exp
diesel exposures using a EuroV engine show even at non-cytotoxic concentrations
induction of oxidative stress response (HO-1, NQO1)

In vitro inhalation models could be valuable for evaluation of the toxicity of combustion emissions
that data from (future) studies can be easily compared and that conclusions are more robust
for regulation; harmonization and validation is needed!
<table>
<thead>
<tr>
<th>Acknowledgements</th>
<th>Institutions</th>
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<tbody>
<tr>
<td>Gröllers-Mulderij, Pieter Hiemstra, Maria Zarcone</td>
<td>LUMC</td>
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<td>Pieter Tromp, Eugene van Someren, Marie Kuper, Frieke Kuper, Cyrille Krul, Ruud Verbeek, Gerrit Kadijk</td>
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<td>Evert Duistermaat, Frederique van Acker</td>
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<td>Arno Gutleb</td>
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<td>Harri Alenius</td>
<td>INERIS</td>
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University of Finland
THANK YOU FOR YOUR ATTENTION!
Problem of current regulatory emission standards: PM mass

Toxicity evaluation based on in vitro models be a solution?

Toxicity screening of Euro V emissions

Conclusion
VALIDITY IN VITRO SYSTEMS

1. Choice of cells
   - Cell lines
   - Primary human lung epithelials cells
   - Induced pluripotent stem cells
   - Mono or co-cultured

2. Culture system used

3. Type of exposure

4. Possibilities for valid read-outs to assess the effect of exposure
Experimental Set-Up

**Oxidative Stress:**
- HO-1

**Inflammation:**
- ICAM-1,
- IFN-\(\gamma\),
- IL-1\(\beta\),
- IL-6,
- IL-8,
- IL-13,
- IP-10,
- MCP-1 multiplex analyses

**Cytotoxicity:**
- LDH,
- TEER

**Genotoxicity:**
- Comet assay

**Gene Array:**
- Illumina beadchip (human HT-12v4)

**System Components:**
- Turn-table dust feeder
- Venturi
- Buffer chamber
- Vitrocell system

**Dilution & Exposure (1h):**
- Air control
- Dilution 1: L
- Dilution 2: M
- Dilution 3: H

**Biological Response (24h post exposure):**
- Aerolizing

**Air Control Dilutions:**
- Dilution 1: L
- Dilution 2: M
- Dilution 3: H

**Particle Compound Analyses:**
- SEM analyses
- MMAD (APS)
- Gravimetric analysis

**Cell Types:**
- A549
- Beas-2B
- MucilAir
- PBEC
EFFECT OF THE EXPOSURE DURATION

6h post exposure

Exposure time:
- 60 min
- 150 min
- 375 min

LDH cytotox
IL-8 protein
HMOX1 gene expr

Charts showing data for different exposure times and concentrations.
INTEGRATED STRESS RESPONSE AND UNFOLDED PROTEIN RESPONSE

- Integrated stress response:
  - aa starvation
  - oxidative stress
  - dsRNA (viruses)
    - GCN2
    - HRI
    - PRK

- Unfolded protein response:
  - Unfolded proteins
    - PERK
    - IRE1α
    - ATF6

- EIF2α phosphorylation
- ATF4 activation
- Inhibition of protein translation
- HMOX1
- CHOP
- GADD34
- XBP1spl
- BiP
INTEGRATED STRESS RESPONSE

Zarcone et al. AJP Lung 311(2016)

60/150/375 min exposure; 6h post exposure

CHOP

GADD34
Workshop ALI in vitro models for respiratory toxicology – Paris 2016

To ensure that data from (future) studies can be easily compared and that conclusions are more robust and useful for regulation; harmonization and validation is needed!

Challenges to overcome:

- What should be validated?
- Lack of standardization between groups
- In vivo human relevance
TNO connects people and knowledge to create innovations that boost the competitive strength of industry and the wellbeing of society in a sustainable way.

‘INNOVATION FOR LIFE’
In public-private collaboration with partners from the golden triangle

Contract research for and with customers

Exploiting knowledge through spin-offs, licences, etc together with other companies
Together with universities in public-private collaboration with partners from the golden triangle

Contract research for and with customers

Exploiting knowledge through spin-offs, licences, etc. together with other companies

TNO - INNOVATION PROCESS

DEVELOPING KNOWLEDGE

APPLYING KNOWLEDGE

TRANSFERRING KNOWLEDGE

23. ETH Conference on Combustion Generated Nanoparticles, Zurich - June 19 2019
PM mass (g kJ^{-1}) emissions for the fuel types and blends tested (6 ETC, 5 ETC and PPO 4 ETC). Error bars indicate the standard error of the mean. *, significally different from B0 at $P < 0.05$, $< 0.01$, $< 0.001$ respectively.

Fig. 5. Relative cytotoxicity for the fuel types and blends tested, in % compared to positive control, 1% Triton-X100. Each bar represents 3 ETC. Error bars indicate the standard error of the mean. *, **, *** significantly different from the mean of B0 at $P < 0.05$, $< 0.01$, $< 0.001$ respectively.
PM mass

Properties of non-volatile exhaust particles

<table>
<thead>
<tr>
<th>Mass conc. (µg m⁻³) (SD)</th>
<th>Number conc. (x10⁶ cm⁻³) (SD)</th>
<th>CMD (nm) (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>485 (14)</td>
<td>2.30 (0.03)</td>
<td>50</td>
</tr>
<tr>
<td>4.6 (0.7)</td>
<td>1.14 (0.04)</td>
<td>18</td>
</tr>
<tr>
<td>335 (8)</td>
<td>2.04 (0.02)</td>
<td>47</td>
</tr>
<tr>
<td>1.3 (0.5)</td>
<td>0.29 (0.01)</td>
<td>17</td>
</tr>
</tbody>
</table>

FA hydroprocessed esters and fatty acid, SD standard deviation, estMₚₑ estimated particle mass according to Jeannet et al.\textsuperscript{28}

PM inflammatory response (IL-8)

Concentration (pg mL⁻¹)

Jet A-1

HEFA

- 85% Thrust
- Ground-idle
- Particle-free

*
EFFECT OF THE EXPOSURE DURATION

6h post exposure

LDH (% of Triton control)

Exposure time
- 60 min
- 150 min
- 375 min

24h post exposure

CXCL8 protein (pg/ml)

Exposure time
- 60 min
- 150 min
- 375 min
EFFECT OF THE POST-EXPOSURE INCUBATION TIME