Cross Evaluating the Effects of a Cerium-Based Diesel Fuel Additive on Exhaust Toxicity with *in vitro* Air-Liquid Interface Cell Exposure Systems of Different Flow Patterns

Penelope K. Baltzopoulou[a], Lynn E. Secondo[b], Akrivi Asimakopoulou[a], Daniel Deloglou[a], Christos Softas[a], Spyros Petrakis[c], Leonidas Chasapidis[a], Eleni Papaioannou[a,d], Nastassja A. Lewinski[b] and Athanasios G. Konstandopoulos[a,c]

[a] Aerosol & Particle Technology Lab., Centre for Research & Technology Hellas (APTL/CPERI/CERTH), Thermi-Thessaloniki, Greece
[b] Department of Chemical and Life Science Engineering, Virginia Commonwealth University, Richmond, VA, USA
[c] Institute of Applied Biosciences , Centre for Research & Technology Hellas (INAB/CERTH), Thermi, Greece
[d] Department of Chemical Engineering, Aristotle Univ. of Thessaloniki (AUTH), Thessaloniki 54124, Greece

21st ETH-Conference on Combustion Generated Nanoparticles,
22nd of June 2017, Zurich

AEROSOL & PARTICLE TECHNOLOGY LABORATORY

CPERI Chemical Process and Energy Resources Institute

CERTH CENTRE FOR RESEARCH & TECHNOLOGY HELLAS

VIRGINIA COMMONWEALTH UNIVERSITY

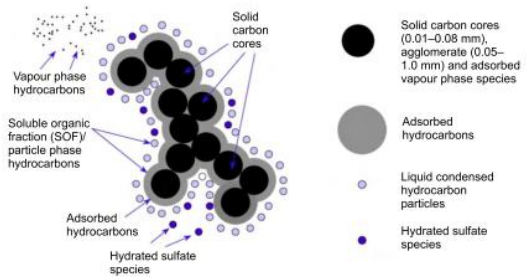
Presentation Outline

- Background on Health Effects of Diesel Exhaust Particles (DEP) & Fuel Additives
- Air-Liquid Interface (ALI) Cell Exposure Systems
- Cellular Exposure Experiments on Diesel Engine
- Biological Endpoints
- Conclusions

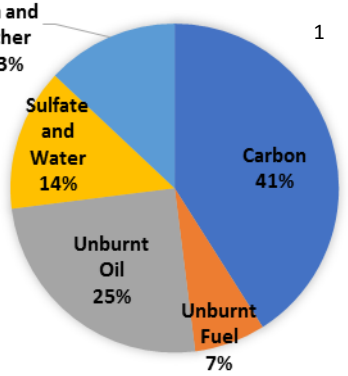
Background: Particle Health Effects

- **Diesel Exhaust Particles (DEP)** are aggregates of carbon, hydrocarbons, PAHs, and unburnt oils[1, 2]

2



1



- **Ce-based fuel additives** decrease the greenhouse gasses & the total particle emissions from combustion

11

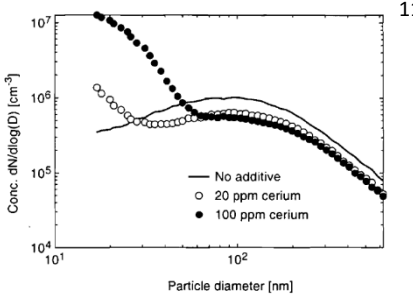
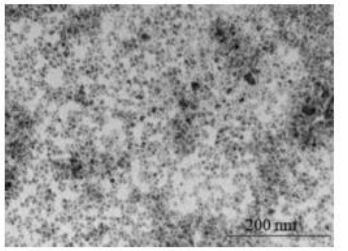


FIGURE 5 Measurements with the SMPS of the L1 engine at 1400 rpm/300 Nm, for different additive concentrations. The emergence of a secondary peak is easily observed

12



TEM of CeO_2 from Envirox fuel additive.

- Diesel Exhaust leads to cardiovascular diseases, respiratory morbidity and lung cancer[3-7]
- Increasing ceria concentrations alter the particle size distribution to a bimodal one attributed both to fragmented soot aggregates and free Ceria NPs[13].

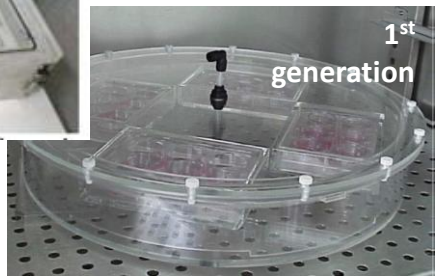
- ***In vitro*** – <u>cytotoxicity, oxidative stress, and inflammatory responses</u> vary in suspension and increase at the Air-Liquid Interface (ALI)[8-10]
- ***In vivo*** – responses <u>indicate cytotoxicity, oxidative stress and lung inflammation</u>
- ***In vitro (ALI)*** – <u>no adverse effects</u>[14,15]
- ***In vivo*** – reported <u>adverse effects</u>: increased macrophage uptake, cell damage, oxidative stress, inflammation[16];

  while <u>reduced atherosclerosis</u>[17]

1. Kittleson J Aerosol Sci. 1998. 2. Twigg et al. Platinum Metals Rev. 2009. 3. Brook et al. Circulation. 2004. 4. Geiser & Kreyling. PFT. 2010. 5. Gorr et al. AJP – Heart Circ Physiol. 2015. 6. Maier et al. Inhal Tox. 2008. 7. Oberdorster et al. PFT. 2005. 8. Tsukue et al. Toxicol in Vitro 2010. 9. Turner et al. Aerosol Sci Tech2015. 10. Cao et al. Am J Respir Cell Molec Bio 2007. 11. Skillas et al., Combustion Science and Technology, 2000. 12. Batley et al. Environ Tox Chem. 2013. 13. Mayer et al. SAE 2010. 14. Fall et al. Nanotoxicology 2007. 15. Steiner et al. Toxicology Letters 2012. 16. Snow et al. 2014. 17. Cassee et al. Env. Research 2012

Cell Exposure Systems

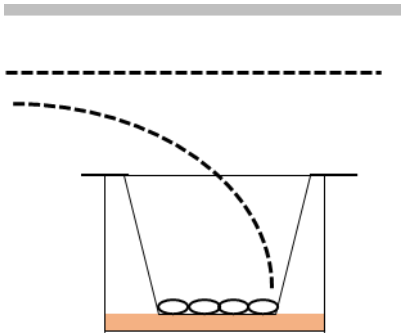
A. Multiculture in-vitro cell Exposure Chamber (MEC II) Asimakopoulou *et al*., 2011



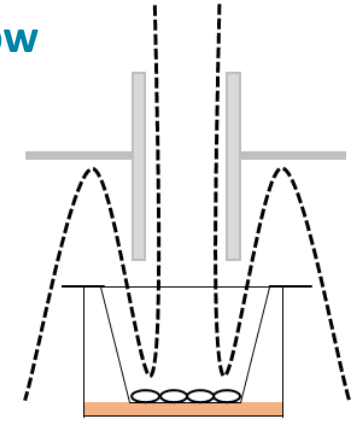
Papaioannou E. et al. (2006) SAE

B. Portable In Vitro Exposure Cassette (PIVEC)
Secondo and Lewinski, in preparation

Parallel Flow



Perpendicular Flow



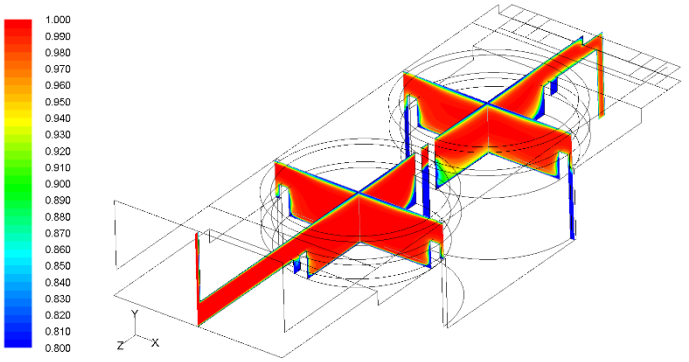
Deposition takes place through impaction, gravitational forces, Brownian motion, and diffusion. Stagnation point flow allows for the distribution of aerosol throughout the system.

Exposure Device with Parallel Flow (MEC II):
Key characteristics

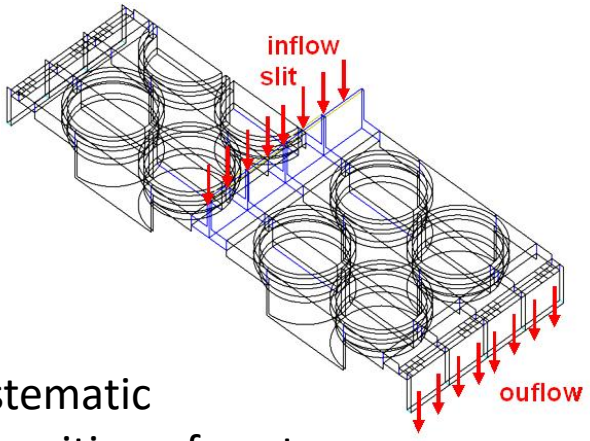
- **M**ulticulture in-vitro cell **E**xposure **C**hamber **MEC II**[1,2,3]

- Sampling device for cell exposure studies simulating the respiratory system.
- The throughput screening possibility is significantly high. MEC accommodates 6 inserts plates (6-well and/or 24-well).
- High degree of flow velocity uniformity

CFD and particle transport simulation



- Visualisation of the soot particles concentration on longitudinal and lateral sections through the cell culture wells *(normalised against inflow concentration)*



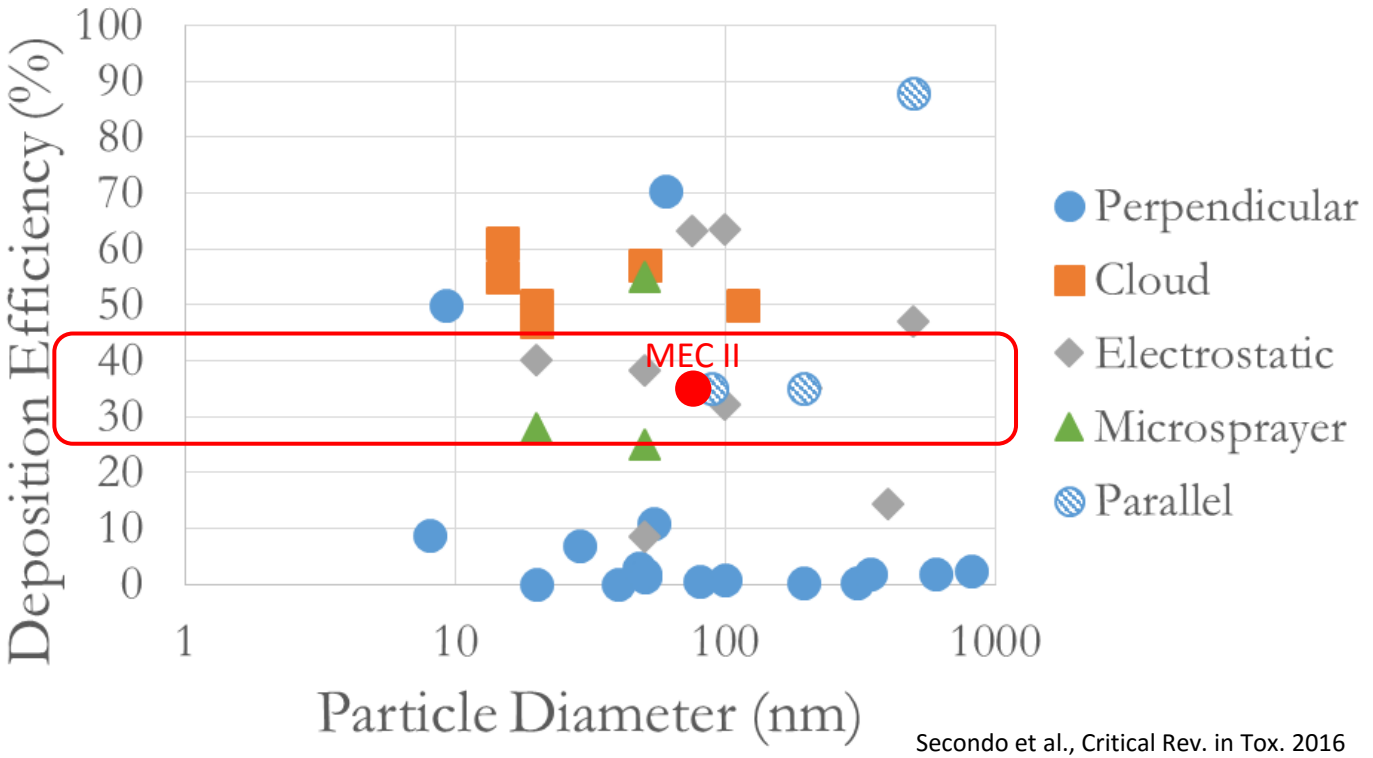
- Systematic deposition of soot particles regardless the location

[1]Papaioannou E. et al. (2006) SAE Tech. Paper No. 2006-01-1075 (SP-2024), 389-399. [2]Asimakopoulou A. et al. (2011) J. Phys.: Conf. Series, 304(1), 012005. [3]Asimakopoulou et al. (2013), J. Phys.: Conf. Series, 429, 012023.

Exposure Device with Parallel Flow (MEC II): Deposition Efficiency

Number Deposition Efficiency ~ **40%**

$$n(\%) = \frac{N_{deposition}}{N_{feed}} \cdot \frac{A_{effective}}{A_{total}} \cdot 100$$

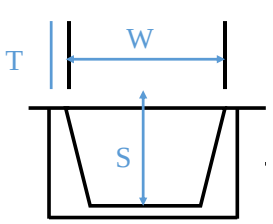


Secondo et al., Critical Rev. in Tox. 2016

Exposure Device with Perpendicular Flow (PIVEC): Motivation

- **P**ortable **I**n **V**itro **E**xposure **C**assette **(PIVEC)**, *Secondo and Lewinski, in preparation*

- Design characteristics:
  1. Highly portable – used at source of emission or in breathing zone
  2. Capture aerosols *in vitro* at ALI
  3. Enclose 6 well and 24 well transwell for deposition
  4. Allow aerosol passage & hold cell media



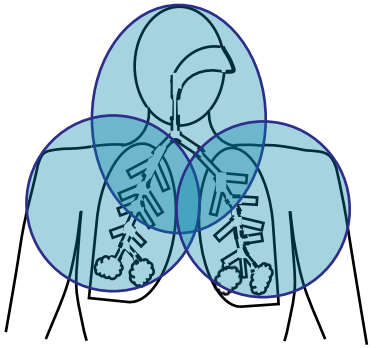
$S/W = 1/2$

$T/W = 0.25$

$W = 37mm$

$T = 9mm$

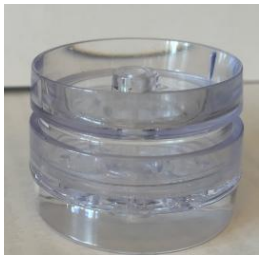
$S = 18.5mm$



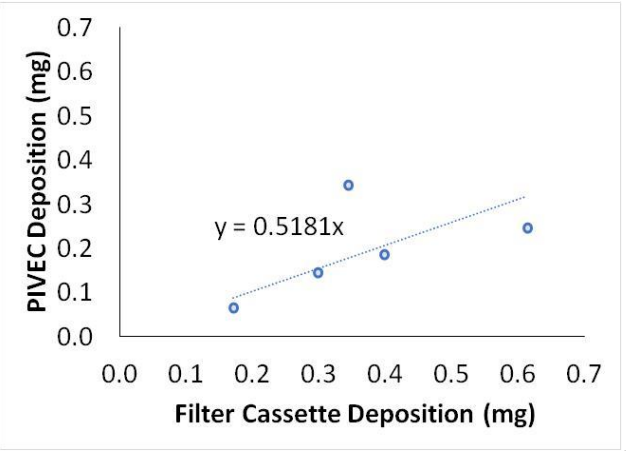
Approximate Breathing Zone

- The PIVEC has been designed as an adaption to the SKC 37 mm filter cassette

PIVEC Original Design



SKC 37 mm filter cassette

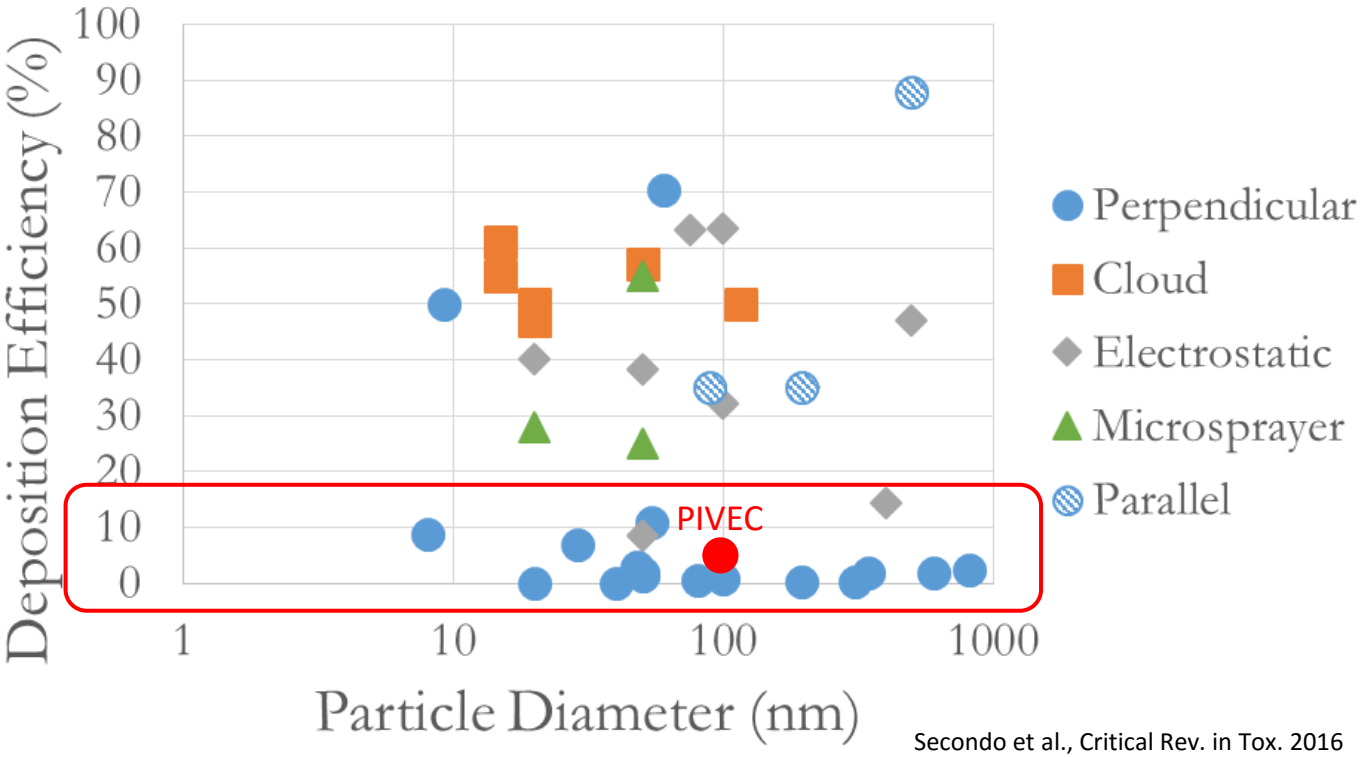


[1] Based on gravimetric measurements performed during acellular 3 hrs exposures on diesel exhaust stream

Exposure Device with Perpendicular Flow (PIVEC): Deposition Efficiency

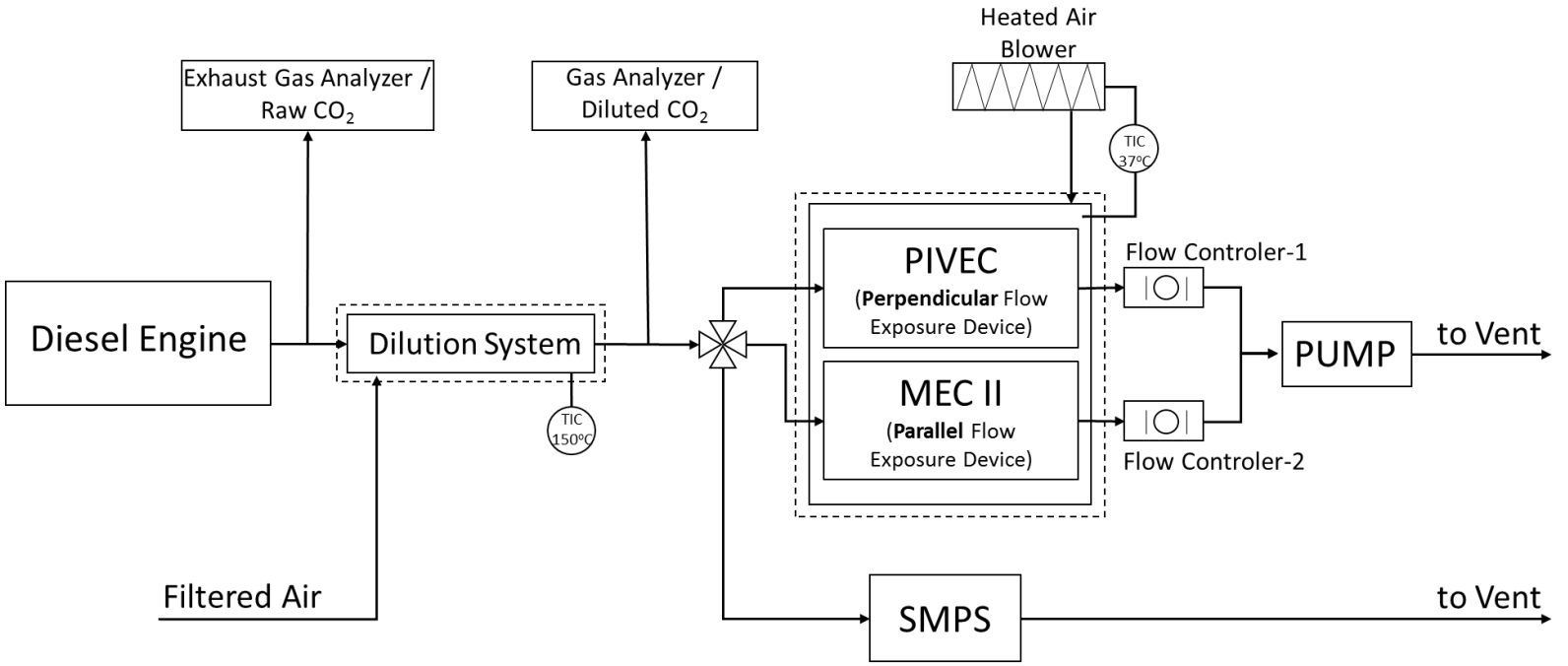
Number Deposition Efficiency ~ **4.2%**

$$n(\%) = \frac{N_{deposition}}{N_{particle}} \cdot \frac{A_{ALI}}{AV} \cdot 100$$



Secondo et al., Critical Rev. in Tox. 2016

Engine Measurements: Experimental Setup



Engine:

- Single Cylinder, four-stroke, air-cooled, direct injection diesel power generator, 5 kW
- Operation Load = 27%

Fuel:

- Commercial Low Sulphur Diesel (LSD) ([S]=6 ppm)
- Ceria-Based Fuel Additive: Envirox ([Ce]=18000 ± 500 ppm)

Exposure Conditions:

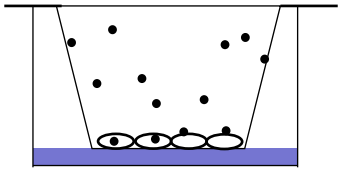
- Diesel Exhaust Particle (DEP) Concentration adjust ~ 1.5 x 10^6 #/cm^3, (Diluted Exhaust, Diluted Ratio ~40-90) (similar to human inhalation: worst case cenario[1])
- Exposure Q = 2 lpm
- Exposure time = 1 hr

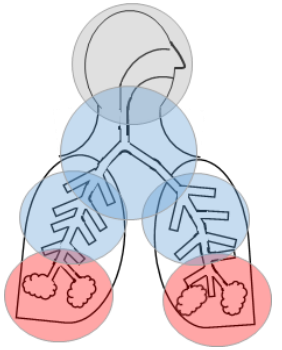
1. Paur , J. Aerosol Sci. 2011

Engine Measurements: Biological protocols

Biological samples:

- Cells cultured at Air-Liquid Interface (ALI)
- A549, adenocarcinomic alveolar cells (quickly growing, often used)





Biological Endpoints:

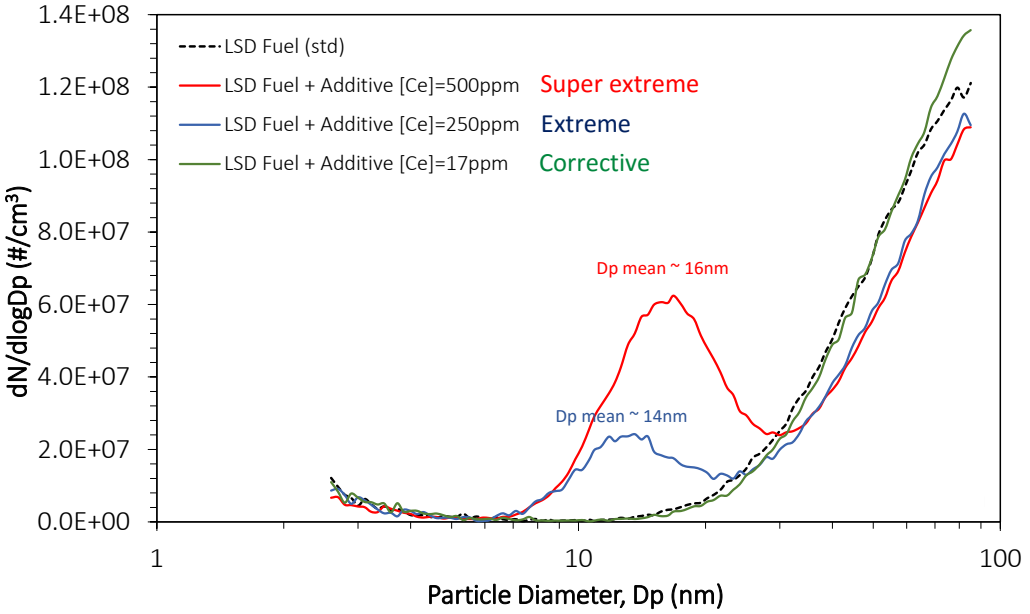
- Cytotoxicity
  - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay from active mitochondria

    (24 hrs Post Exposure / Incubated in 37°C, 5% CO_2, 80% humidity)
- Oxidative Stress
  - 2',7'-dichlorofluorescin diacetate (DCFH-DA) generation as intracellular Reactive Oxygen Species (ROS) generation

    (2 hrs Post Exposure / Incubated in 37°C, 5% CO_2, 80% humidity).

Exposure Exhaust Characterisation 1/2

Engine's behavior on the Ce-based additive (ENVIROX) addition:



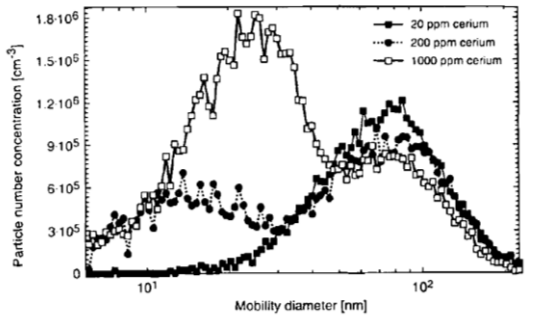


FIGURE 7 Soot particle size distribution calculated for the undiluted exhaust of the YAMAHA engine at 3 kW. The additive peak is dominant for additive dosage above 200 ppm

Skillas et al., Combustion Science and Technology, 2000

Cases studied:

	Case I:	Case II:	
	LSD	LSD w. ENVIROX	
LSD[1] Sulphur Content (ppm)[1]	6	6	
Ce-based additive concentration (ml/lt fuel)	0	1	
CeO_2 content in the final fuel mixture [2] (ppm)	0	17 ± 0.8	
Additive use	-	**"corrective"**	*Real world scenario*

[1] Commercial Low Sulfur Diesel (LSD)
[2] Measured by Inductively Coupled Plasma (ICP) mass spectrometry as Ce and assuming that all Ce appears as CeO_2
[3] According to manufacturer's directions for Diesel Particle Filter (DPF) de-blockage

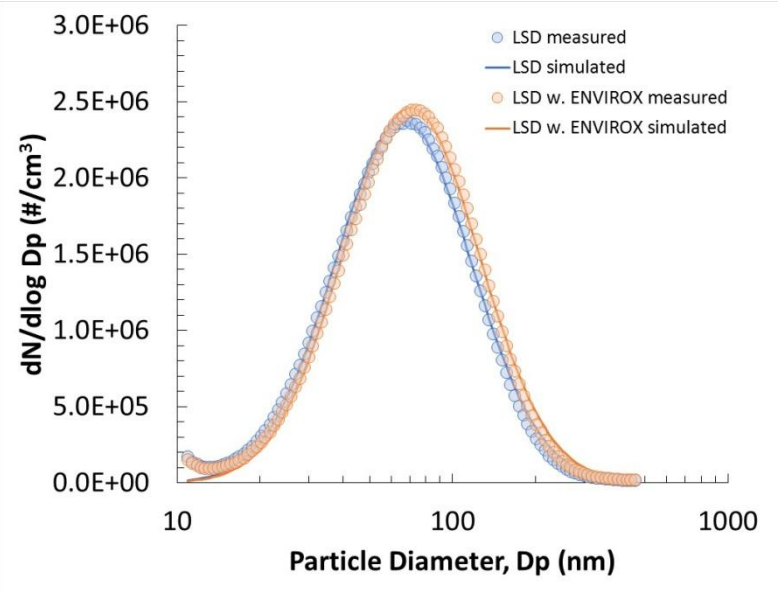
Exposure Exhaust Characterisation 2/2

Exposure Particle Emissions [1] :

	Case I:	Case II:
	LSD	LSD w. ENVIROX
Particle Diameter		
Mean (nm)	80 ± 5	82 ± 3
Geometric Mean (nm)	71 ± 5	72 ± 3.5
Particle Concentration		
Number Concentration (#/cm³)	1.50 E+06	1.54 E+06
Coefficient Variation (%)	4.4	3.5

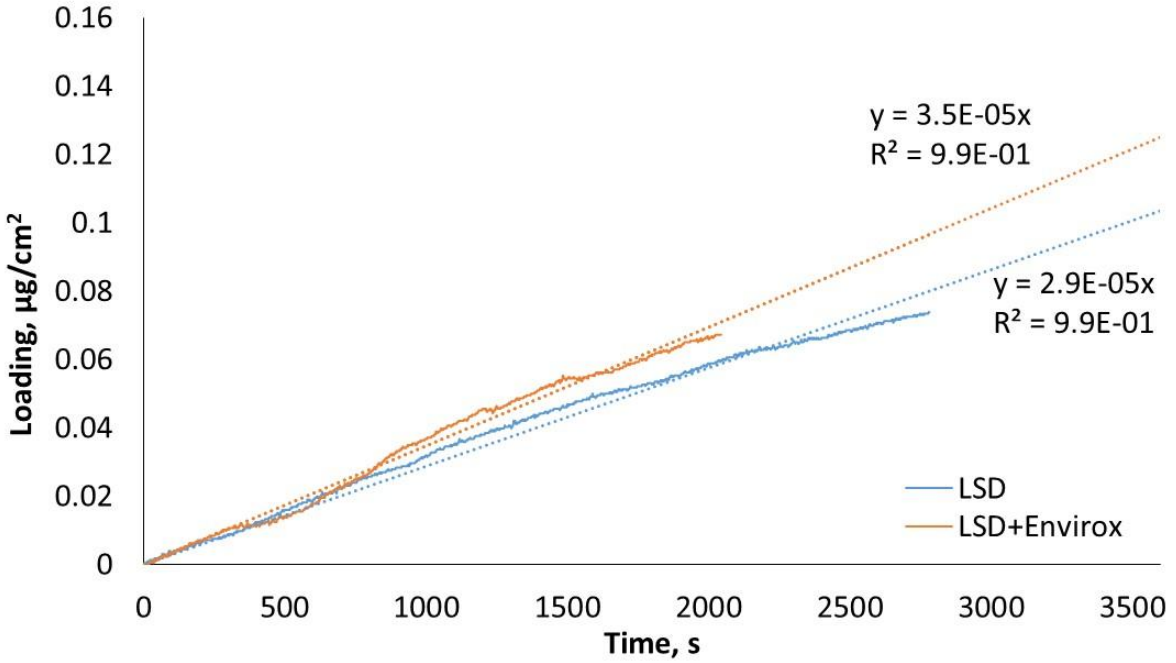
[Ce] = 17ppm ± 0.8 ppm

[1] Measured by the Scanning Mobility Particle Counter (SMPS, TSI Inc.)



Exposure Device with Parallel Flow (MEC II):
Dose determination

- **Q**uartz **C**rystal **M**icrobalance (QCM): (quasi) real-time dose measurements
- A QCM sensor is placed in MEC instead of the insert and exposed to the aerosol in the same way as the cell cultures



Loading of soot particles from LSD and LSD with ENVIROX additive is **0.104** and **0.126 µg/cm²/h**, respectively

Difference in slopes is attributed to higher density of ceria (7.2 g/cm³) compared to soot (2 g/cm³)

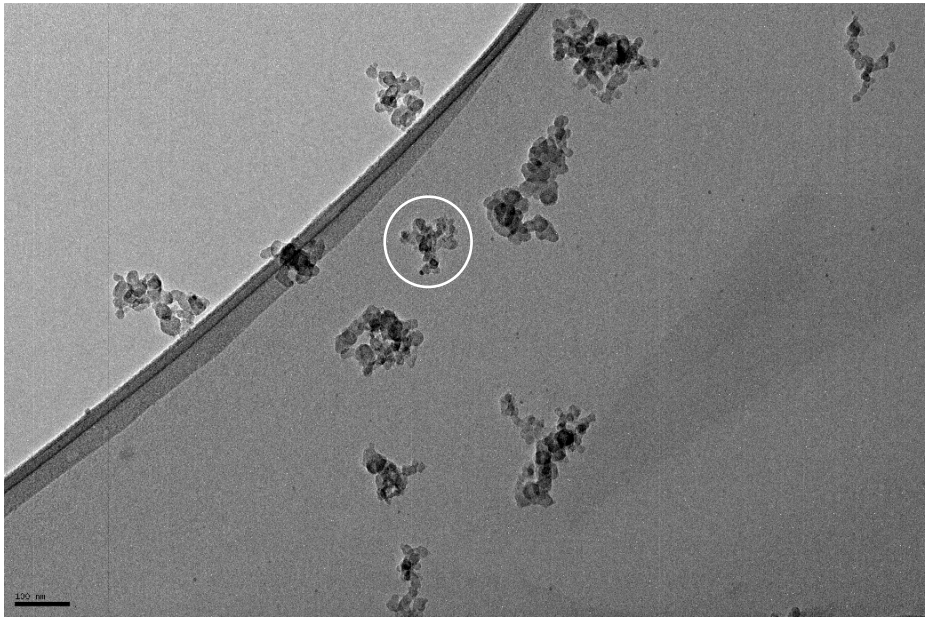
Results agree with published data of cell exposure to nanoparticles (50-500nm) in similar exposure systems[1] and correspond to the accumulated daily dose of human inhalation worst case scenario (i.e. 0.12 µg/cm²/day)[2].

1. Mülhopt et al. J. Phys. 2009. 2. Paur et al. J. Aerosol Sci. 2011

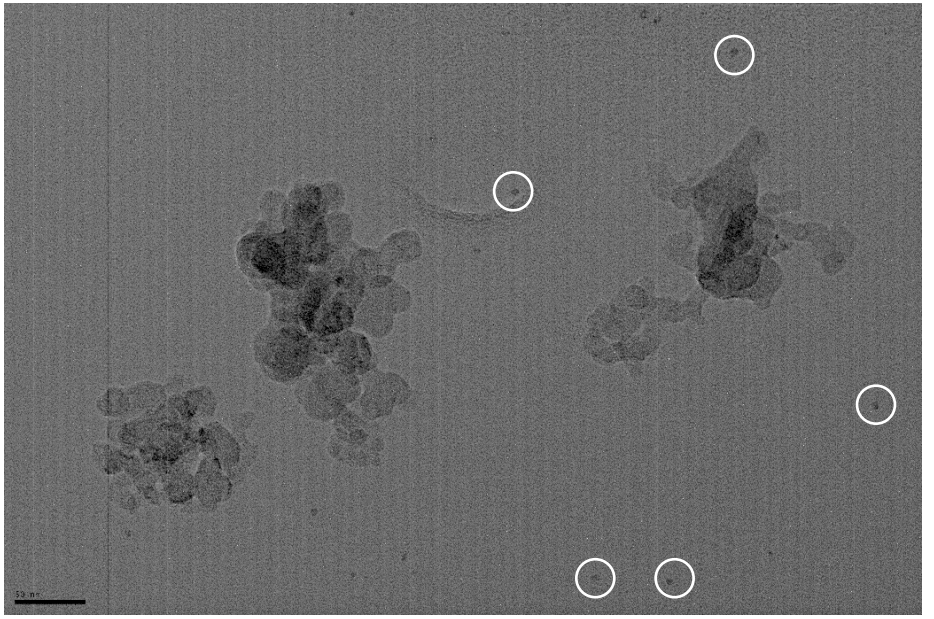
Exposure Device with Parallel Flow (MEC II): Particles deposition

- **T**ransmission **E**lectron **M**icroscopy (TEM):

Samples collected from several positions in MEC for 1hr of exposure to LSD with Envirox



Soot particles
(average aggregate diameter = 80nm)



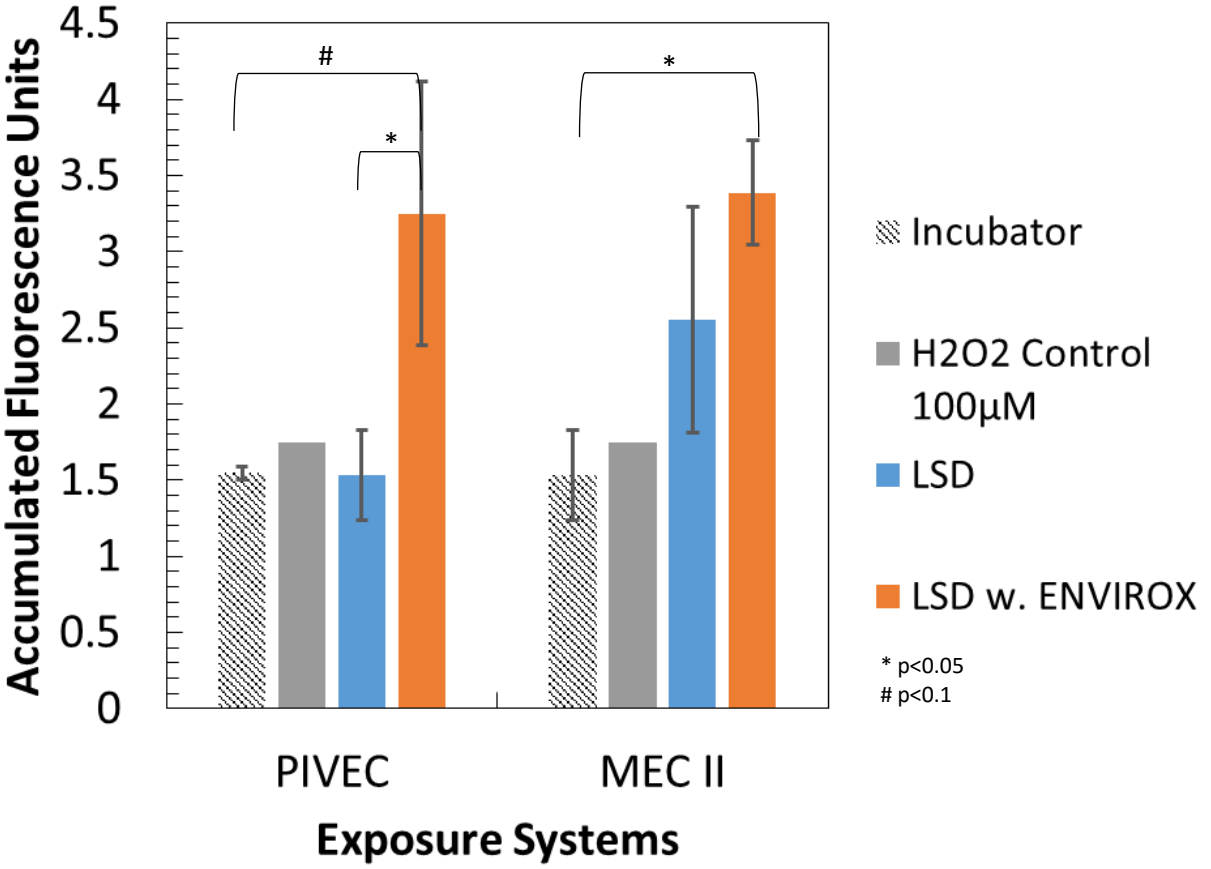
Soot and Ceria particles
(average particle diameter = 5nm)

Assuming that the cell number per surface area is $3 \cdot 10^5$ cells/cm^2 (typical cells population on 24 well insert), average deposition density of soot particles is **3,000 #/cell/h**, which corresponds to ½ of the accumulated daily dose of human inhalation worst case scenario (i.e. 6,700 #/cell/day[1]).

1. Paur et al. J. Aerosol Sci. 2011

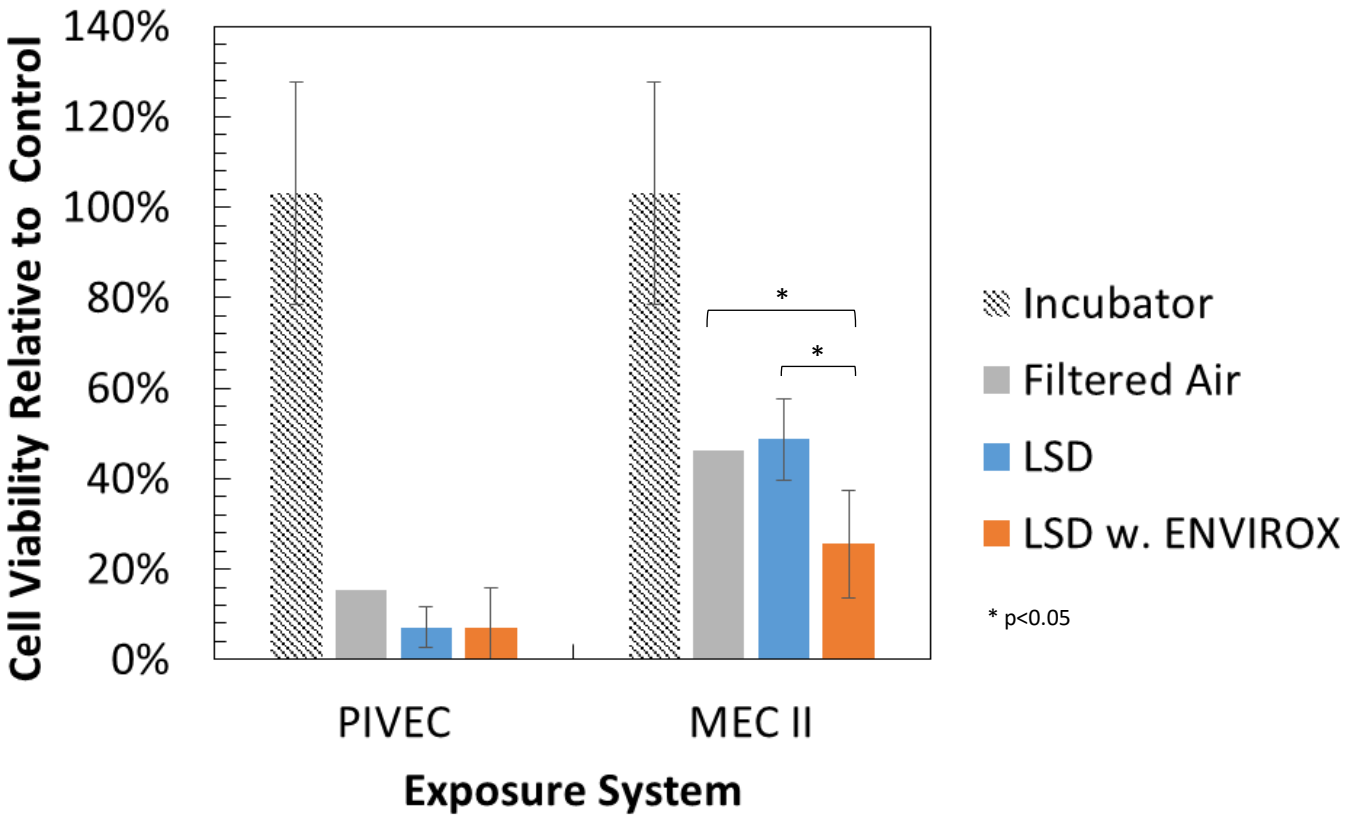
Cellular engine exposure: Oxidative Stress

- Increase in ROS can lead to stop in growth cycle, apoptosis, or even necrosis.
- Not all increases in ROS lead to cytotoxicity



- Both systems show <u>increased ROS generation</u> when using LSD w. Envirox (1ml/lt additive; corresponding to 17ppm CeO_2 content).
- Cellular exposures with the **parallel flow** exposure system (**MEC II**) indicate:
  - <u>No statistically significant difference</u> when comparing LSD vs LSD w. Envirox (no additive effect)
- Cellular exposures with the **perpendicular flow** exposure system (**PIVEC**) indicate:
  - <u>Increased ROS generation</u> when comparing LSD vs LSD w. Envirox (additive effect)

Cellular engine exposure: Cytotoxicity



- Cellular exposures with the **parallel flow** exposure system (**MEC II**) indicate:
  - Increased cytotoxicity of LSD w. Envirox. relative to the filtered air (same trend as ROS generation).
  - Increased cytotoxicity when comparing the LSD vs LSD w. Envirox (additive effect)
- Cellular exposures with the **perpendicular flow** exposure system (**PIVEC**) indicate:
  - No statistically significant difference of LSD or LSD w. Envirox. relative to filtered air
  - Cells are influenced by the gas flow.

Conclusions 1/3

- Dose determination at the parallel flow exposure system (MEC II) based on QCM method & based on SoA particle number counting corresponds to the **accumulated daily dose of human inhalation worst case scenario**.

- Cellular exposures with the **parallel flow** exposure system (**MEC II**) indicate:
  - Adverse health effects (oxidative stress & cytotoxicity) in the case of LSD w. Envirox additive (17ppm CeO_2).
  - *Fuel Additive effect* (LSD vs LSD w. Envirox) on cytotoxicity

- Cellular exposures with the **perpendicular flow** exposure system (**PIVEC**) indicate:
  - Adverse health effects (oxidative stress) in the case of LSD w. Envirox (17ppm CeO_2).
  - *Fuel Additive effect* (LSD vs LSD w. Envirox) on ROS generation
  - No biological relevant effect on cytotoxicity due to high cell influence caused by the background filtered air.

Conclusions 2/3

- The two studied Air-Liquid Interface cell exposure systems show differences on the biological assessment of the Diesel Exhaust Particles with and without Ce-based fuel additive probably due to their **different flow patterns that mimick different particle deposition and cause a different degree of stress**.

- The flow pattern is a design choice depending on the scope / motivation of each exposure system; so one should compromise between system's efficiency and application:
  - **MEC II** is designed for **high deposition efficiency** and **high-throughput screening of nanoparticle toxicity** (contributing to identified gap in the field)
  - **PIVEC** is designed for **portable / personal sampling** (contributing to occupational health studies and to the *exposome* concept)

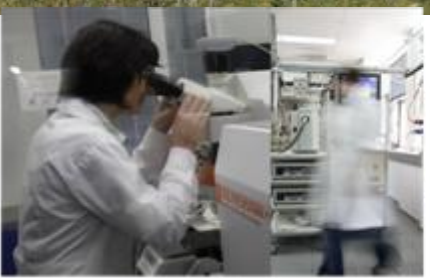
Conclusions 3/3

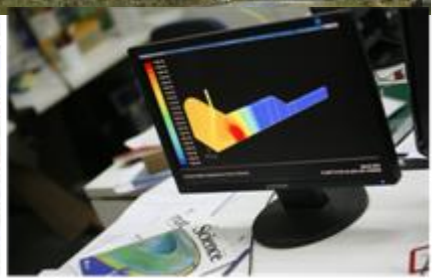
- Inhalation exposure remains an important field of study but still with a lot of challenges on the correlation among *in vitro* results due to different cell exposure techniques; that is also the case for the fuel additives health impact assessment.

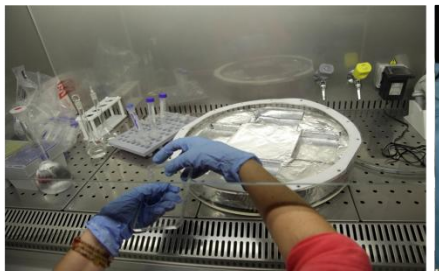
- Fuel additive adverse health effects were observed, despite the unaffected on particle size distributions. Such effect could be attributed also to the free ceria nanoparticles (d < 10nm) (….a small contributing to the open discussion about the regulation of sub-23nm particle vehicle emissions).

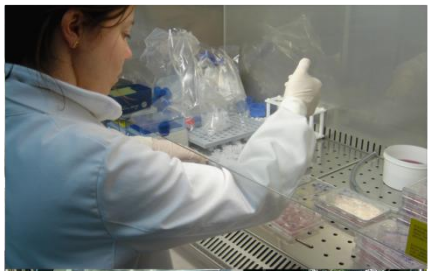
Acknowledgements

- Lynn E. Secondo
(VCU PhD Candidate supervised by Dr. Nastassja Lewinski)
- Fulbright Program
- My colleagues at the APT Laboratory (CPERI/CERTH)
- SUREAL-23 project (H2020 project, G.A. No. 724136)









Thank you for your attention!

AEROSOL & PARTICLE TECHNOLOGY LABORATORY

pbaltzop@cperi.certh.gr
http://apt.cperi.certh.gr