in vitro and in vivo Methodologies for the evaluation of cardiorespiratory impact of complex aerosols : application to combustion engine emissions.

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Inhalation experimental studies consist essentially to expose mice, rats or hamsters to continuous flows of diluted Diesel engine exhausts in closed chambers or in nose o,ly exposure tubings. Exposure PM concentration ranges are 0.1 to 12 mg/m3 for 6 to 16 hours daily, 5 to 7days a week for 12 to 24 months. (EPA, 2000). These dosages represent a massive exposure, compared to the in vivo human situation.
Mauderly et al. 1986 showed that rats exposed for 2 years at PM (.35 to 7.1 mg/m3) dosages which induce high lung burden, develop tumors. Heinrich et al. 1986 and Iwai et al. 1986 clearly showed that filtered exhaust only induced very limited effects thus showing the preponderant role of PM in the observed effects. These authors also showed that similar exposure to carbon black induced the same tumor occurrence in rats. Finally, it is important to state that the rat is the only species where these tumors could be experimentally induced by combustion PM and carbon black. (no tumors in CD-1 mice Mauderly et al. 1996, in hamsters Heinrich et al. 1986, nor in monkey Lewis et al. 1989).
The study of DNA adducts after chronic inhalation exposure to Diesel soot (PAH 30%), carbon black(PAH O.4%) and titanium oxide (PAH 0%) 6.2 mg/m3 showed that higher levels of adducts were detected (x4) in pneumocytes II of exposed rats (Bond et al. 1990), these adducts were of similar chemical composition after exposure to the 3 PM types and did not correspond to PAH adducts (Gallahger et al. 1989). Nilsen et al. 1999 recently showed that these adducts might be related to small MW oxygenated compounds, mainly aldehydes like 2-hydroxyalkyles, ethene or ethylene oxide adducts on N-terminal valine of hemoglobin. It should be pointed out that these authors did not report PAH adducts on hemoglobin after inhalation in vivo.

Finally, Iwai et al. 2000 report that erly oxidative lesions induced by rat exposure to Diesel emissions coupled with prolonged inflammatory reaction may represent an important trigger for the late carcinogenesis observed in aging rats after a long lag phase like suggested previously by Driscoll et al. (1996), for silica or carbon black.

In their study, Nilsen et al. al. 1999, in either ginuea pig or mice inhaling Diesel ehausts with soot concentration of 8mg/m3 8hours a day for two weeks, failed to demonstrate any EROD induction in lung tissue. Since EROD activity is most sensitive to PAH inducing effects, it appears to be most probable that PAH are not made biodisponible in lung tissue after inhalation of Diesel PM.

Instillation experiments consist in the intratracheal injection of a concentrated suspension of particles under light anesthaesia to rats, mice or hamsters. PM concentrations range from 20 to 80 mg/ml, injection volume ranging from 50 to 200 µl. The high acidic nature of PM due to the presence of sulfuric acid makes it necessary to prepare the suspension in buffered solution pH 7.4, the poor miscibility of PM to water necessitates the use of tensioactive agents : Tween 20

or 80, Lecithins or solvents like DMSO, which may desorb some water-unsoluble components from PM.

In rat instillation studies, Iwaï et al. (1997) and Dasenbrock (1996) demonstrated a clear role of PAH in the induction of lung tumors. Savela et al. (1995) have clearly identified PAH DNA adducts in in vitro and in vivo experiments with Diesel PM extracts.

Ohyama et al. 1999 showed that NO2 and SO2 were capable of promoting activity on the carcinogenic potential of Diesel PM extracts. These authors showed that PM extracts alone did not increase tumor formations but that alveolar adenomas and carcinomas development was clearly observed after co-exposure with NO2 and SO2.

Finally recent in vitro evidences showed that effects did not differ when soot suspension were filtered or not before exposure, which support that PAH biodisponibility is clearly modified during the suspension preparation procedure making them available for DNA adduct formation and PAH specific toxicity like p450 induction.

In order to better understand the difference of toxicity patterns recorded between inhalation and instillation exposure, Osier and Oberdorster made a direct comparative study where they showed that in instilled animals a high increase in BAL macrophages, polynucleated cells which was barely observable in inhaled animals. PM lung distribution was more homogeneous after inhalation (more than 80% of alveoli) than after instillation (less than 30% of alveoli), and alveolar macrophages were much more overloaded with PM after instillation than after inhalation. These results have been confirmed by Suarez et al. 2001 in guinea pigs.

These observations are of great incidence to point out to the risk associated with very local high PM and desorbed PAH burden after instillation of massive doses which may not occur after inhalation and thus may explain the differences in toxicity patterns observed between inhalation and instillation experiments.

Authors would like to point out to the fact that the same statements may explain the discrepancies of results obtained after bi-phasic air liquid in vitro systems compared to medium suspended PM models for which massive concentrations of soot in the presence of tensio active agents are again used, regardless of any relevance to the in vivo lung particle dosimetry (deposition and clearance rates).

In the view of these observations, we have developed exposure systems to continuous flows of diluted engine emissions for both in vitro (organotypic cultures of rat lung tissue)(Morin et al. 1999, 4th and 6th ETH conference on combustion particles, Bion et al. 2002) and in vivo cardiorespiratory experiments (inhalation on vigile unconstraint rodents)

Excellent correlation have been observed between in vitro and in vivo lung toxicity patterns which confirm the pertinence of the short term response in vitro model for assessing health impact of new development of engine technologies and emission after treatment strategies.

Recent developments in the field of automated ECG analysis in rodents by telemetry techniques made possible the study of the Diesel emission impact on both healthy and chronic heart failure rats. These experiment clearly show that healthy rat electrocardiogram is barely affected by exhaust exposure but a small

decrease in heart rate variability, while in chronic heart failure rats, within 15 to 30 minutes of exposure, arrhythmia episode frequency clearly increased and lasted for at least 6 hours after a 3 hour exposure. These episodes consisted mainly in premature ventricular extrasystoles, bigeminy, unsustained and sustained ventricular tachicardia episodes. A slight decrease in heart rate variability seems to occur but is made difficult to charaterize due to excessive arrhythmia episodes.

These results on cardiac impact of Diesel emission inhalation clearly show that chronic heart failure makes rats much more sensitive to pollutant exposure, which closely mimicks the in vivo human situation.

In conclusion, pertinent tools and technical knowledge are now available to design new experimental models for allergy and mutagenicity due to continuous exposure to complex aerosols which represent the core of the late 5th European framework program MAAPHRI (Multidisciplinary Approaches to Airborne Pollutant Health Related Issues)

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Objectives :

Development of pertinent toxicity models for screening the potential health benefits of new depollution strategies using a global approach of exhaust emissions

Contribution to the development of depollution strategies and regulations with a health / toxicity based rationale

Inhalation / Instillation
Bi-phasic air liquid / Medium suspension

Conflicting Toxicity patterns in Experimental in Laboratory Animals

Conflicting Toxicity patterns In in vitro cell culture systems

Tumor Induction data ?
DNA adduct Formation ?
Inflammation ?
Oxidant Stress ?
P450 Induction ?

Exposure dosages ?
Physicochemical properties ?
Tissular soot deposition ?

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Mode of Exposure
Lung Tissue Reactivity Pattern

In vivo Inhalation

Low phagocytosis rate

80 % alveolar macrophages contain particles

DNA adducts = non PAH

No Difference TiO_2, Carbon Black and Diesel

■

In Vivo Instillation

Phagocytosis rate x15

70 % alveolar macrophages devoid of particles

DNA adducts = PAH

Differences TiO_2, Carbon Black and Diesel

Major Discrepancies Between Instillation and Inhalation Exposures

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Preparation of aqueous soot suspensions for in vivo Instillation or in vitro exposure of cell cultures

Requires the use of tensio-active agents or solvents
vigorous agitation an ultra-sounds

Soot desorption of lipophillic components by tensio-active agents
Lecithins, Tween 20, Tween 80 (up to 0.1%)
Several studies show that soot removal from suspension
does not affect the toxicity response pattern

Tensio-active agents may alter cell barrier properties
Alteration of cell membrane fluidity (active transport capacity)
May facilitate intracellular uptake of lipophillic pollutants

Pollutant bioavailibility
Modulation of toxicity responses

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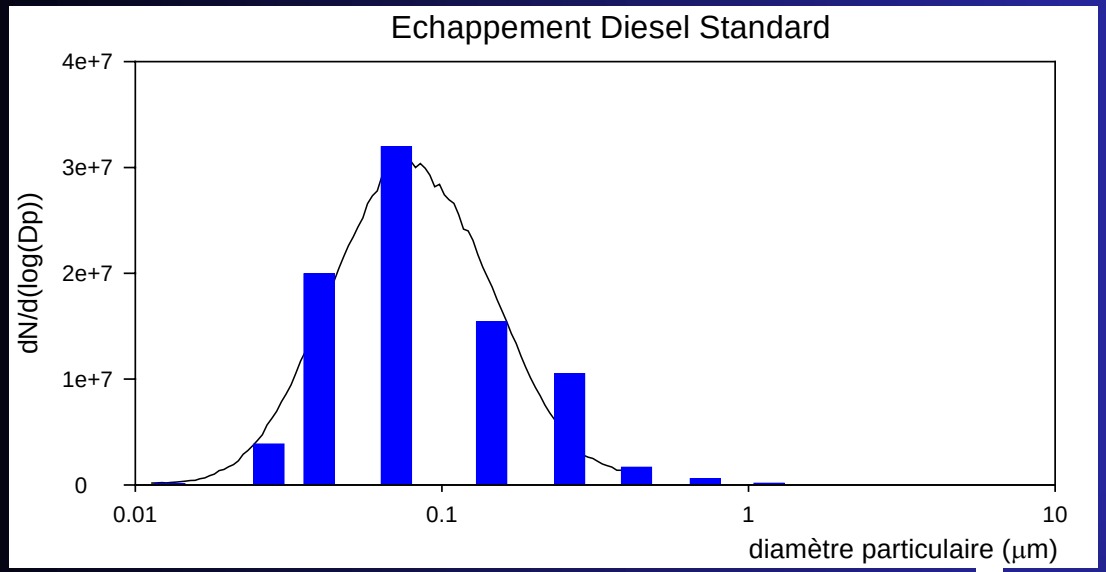
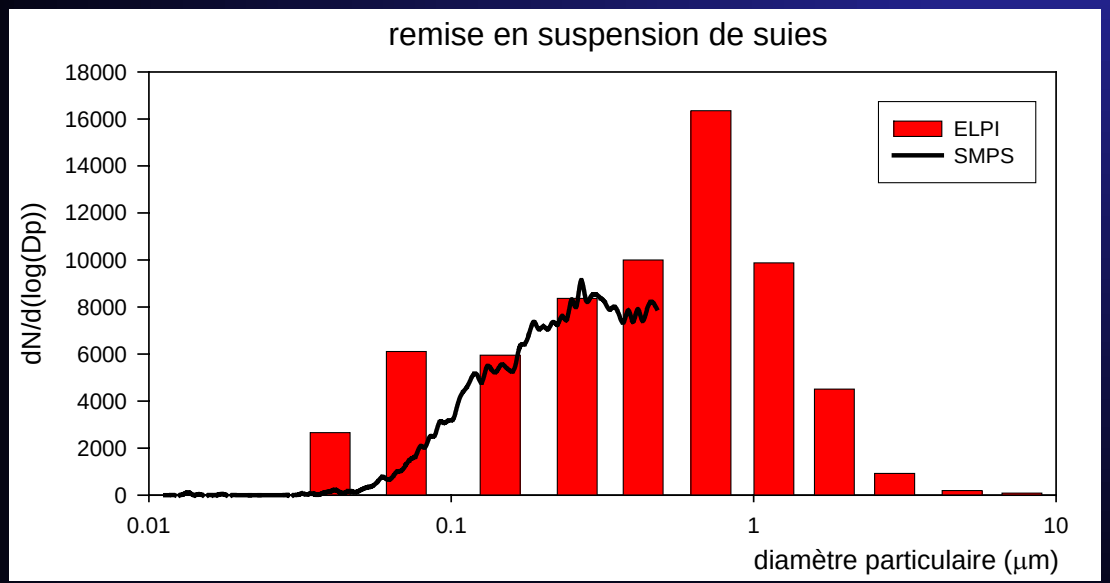
Sampling technique and Soot Size Distributions



ELPI and SMPS
Exhaust Aerosol



ELPI and SMPS
Soot harvested on filters
Resuspended in air

Influence on soot surface density, on macrophage reactivity and phagocytotic rates

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Soot Exposure Dosage Extrapolation

Inhalation *in vivo* :

Rodent in vivo — 0.5 to 10 mg/m^3 8 hours/day

Single administration Instillation *in vivo* :

4 mg/rat = 16 mg/kg Human 70 kg 1.12 g

Equivalent : **28 m^3** exhaust 40 mg/m^3

3,5 years breathing 100µg/m^3

Bi-Phasic models *in vitro* :

Organotypic Cultures — 1 to 10 mg/m^3

Cell lines — 0.5 to 5 mg/m^3

Concentration for Suspensions *in vitro :*

100 µg/cm^2 of cell monolayer

Human lung surface area = 150 m^2 = 1,5 10^6 cm^2

Equivalent : **150 g** particles uniformly distributed on human alveolar surface area

The Debate

in vivo **Inhalation / *in vivo* Instillation**

Bi-phasic models
Air / Liquid *in vitro*

/

PM resuspended in
aqueous solutions *in vitro*

The right way for designing pertinent toxicity tests

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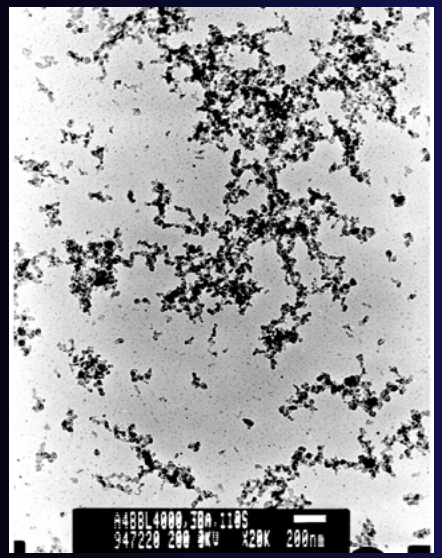
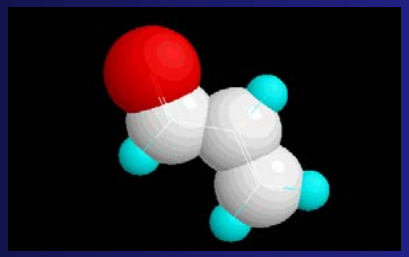
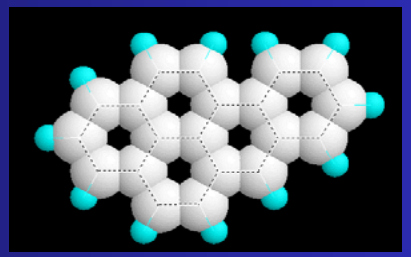
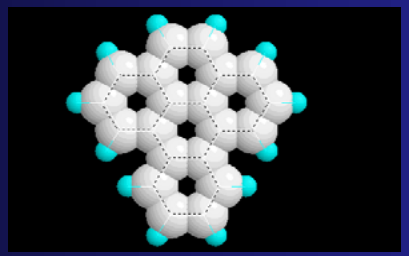
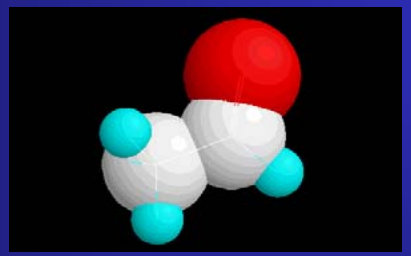
Impact of Complex Aerosols on Lung Tissue in vitro

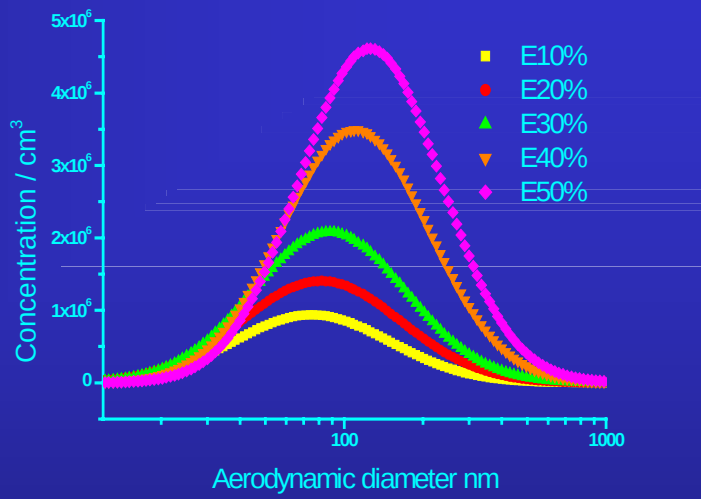


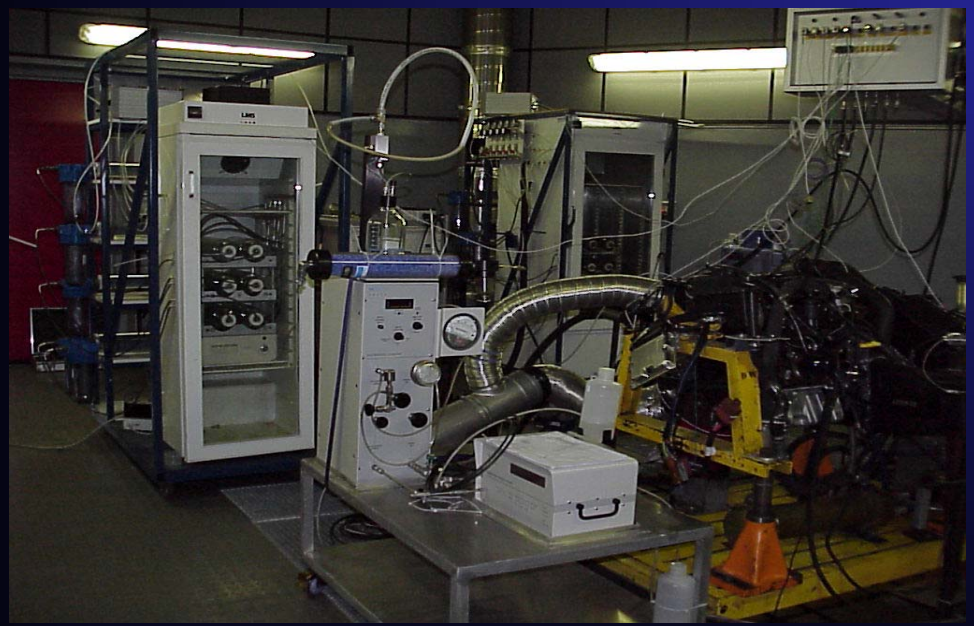


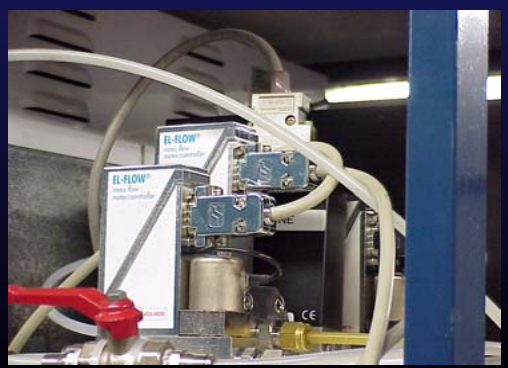


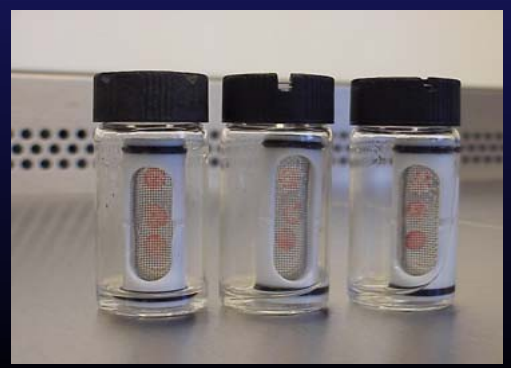












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Advantages of Sampling and Exposure Systems

* Global Approach of Exhaust impact

* No Alteration of pollutant Bioavailibility

* Interactions Aerosol/Biological sample mimicking the in vivo situation (sedimentation and diffusion)

*** No alteration of both gazeous phase and PM physicochemical properties**

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Diesel Exhaust impact (Low NO_2/NO ratio)

	Total	Filtered
GPx	+	+
Catalase	+	+
GSH	---	-
Surfactant	-	-
Polyamines	=	=
TNFα	++	=
Nucleosomes	++	=
TUNEL	+++	=
DNA Ladders	++	=

The Toxic Impact of PM is Modulated By the Oxidant Potential of the Gazeous phase

Inflammation and Oxydant Stress, no Cytotoxicity, Induced by PM at low NO_2/NO ratio (<0.1)

Oxydant Stress, Cytotoxicity and Abolition of Inflammation Due to the Gazeous Phase For NO_2/NO ratio > 0.2

NO_2/NO ratio has been chosen as a marker of exhaust global oxidant potential

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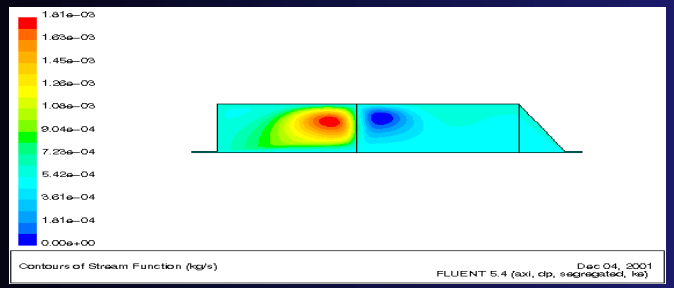
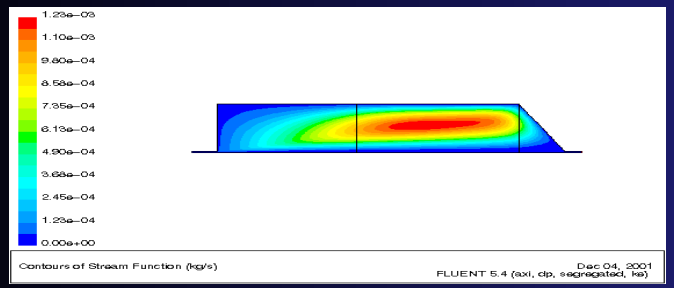
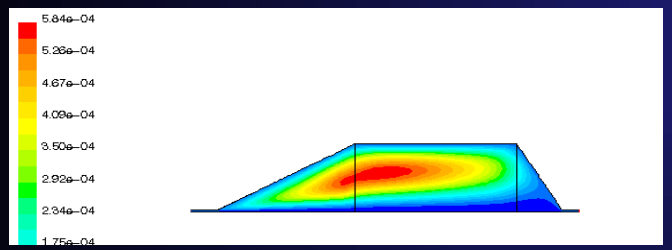
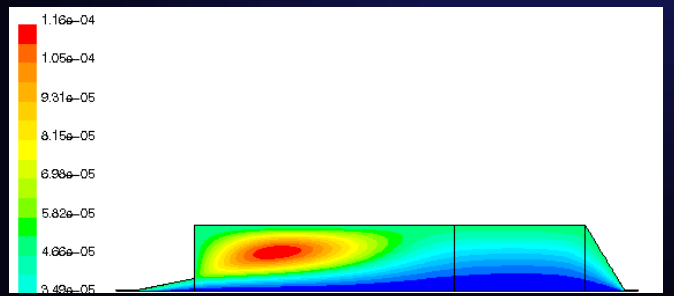
Design of Inhalation Cages for Vigile Unconstraint Rodents

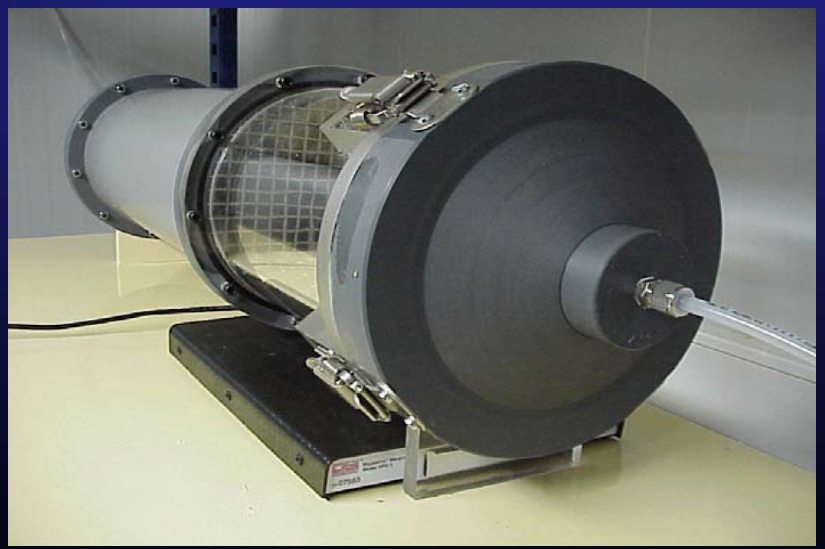
Modelization with FLUENT 2D











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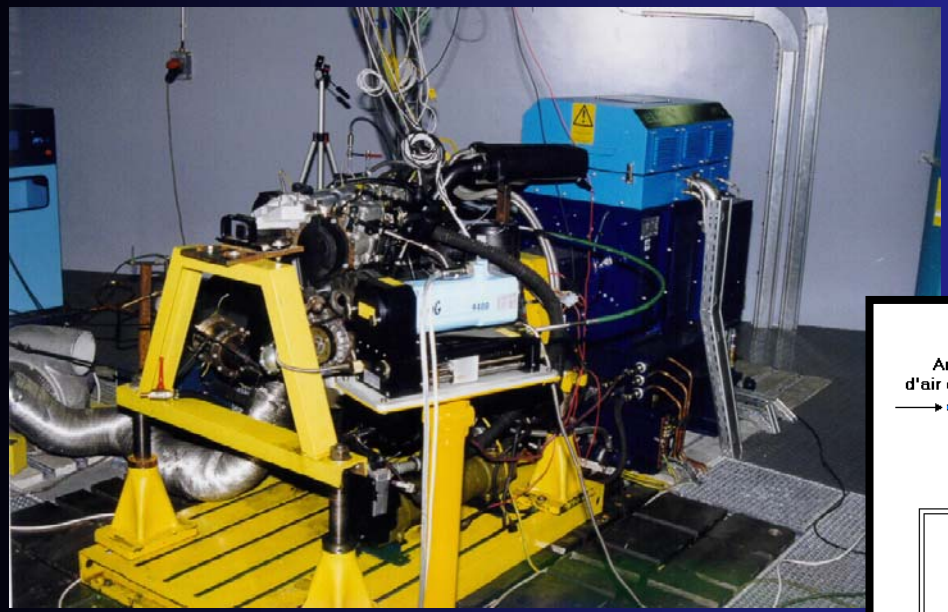
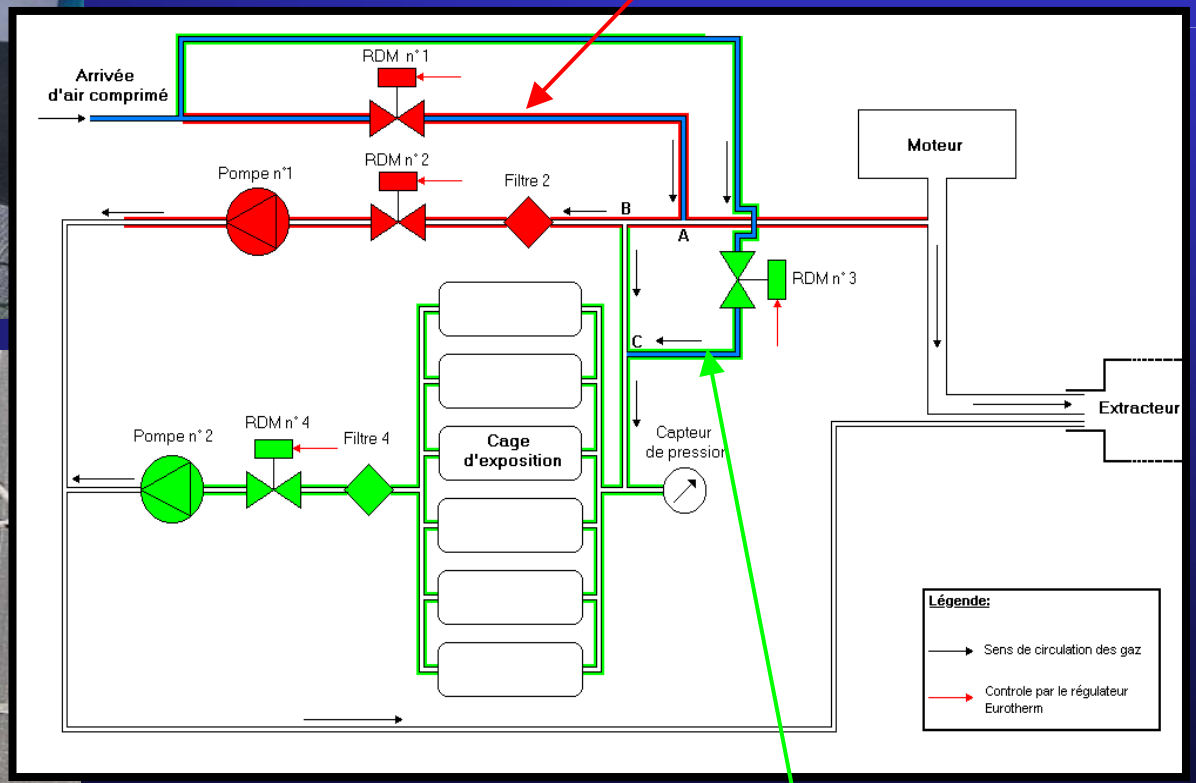
Sampling and dilution of Engine Exhausts



1rst dilution loop



2nd dilution loop

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Experimental Design
In vivo Lung Investigations

Exposure duration 3hour/day for 3days (Exhaust 1/50)

Raw Exhaust Pollutants :

NO 306, NO_2 20, HC 515, Smoke Index 1.29,
PM 3.7 $10^7/cm^3$, 25.8 mg/m^3, mad 106 nm

At necropsy :

Bronchoalveolar lavage
Lung tissue sampling for biochemical and pathology analysis
Culture of lung slices for 3 hours (TNFα)
Plasma sampling for systemic TNFα assay

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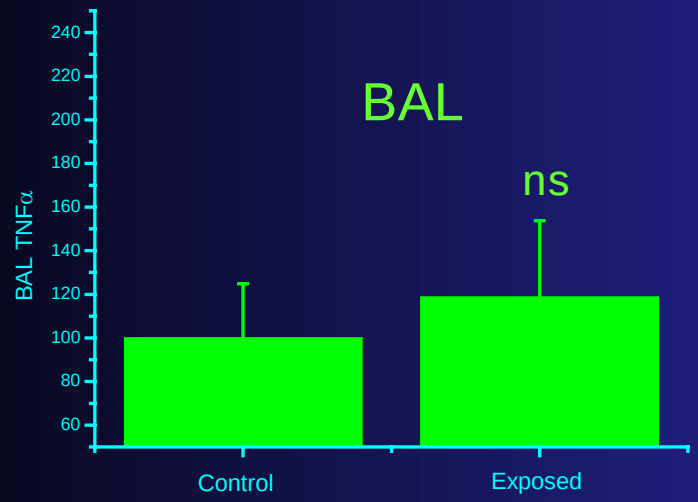
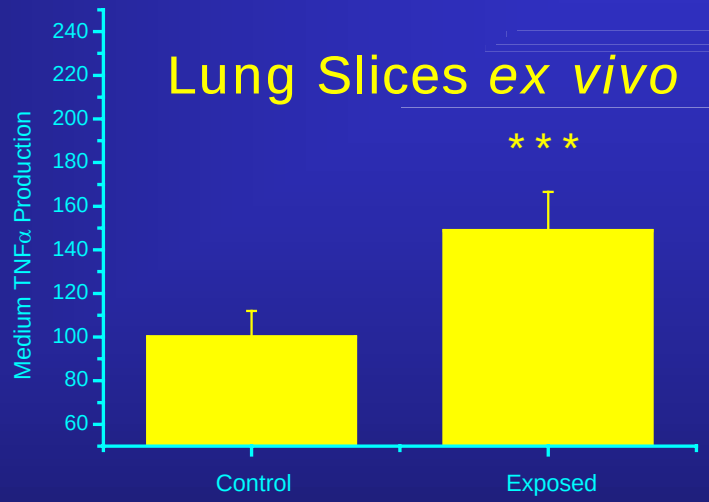
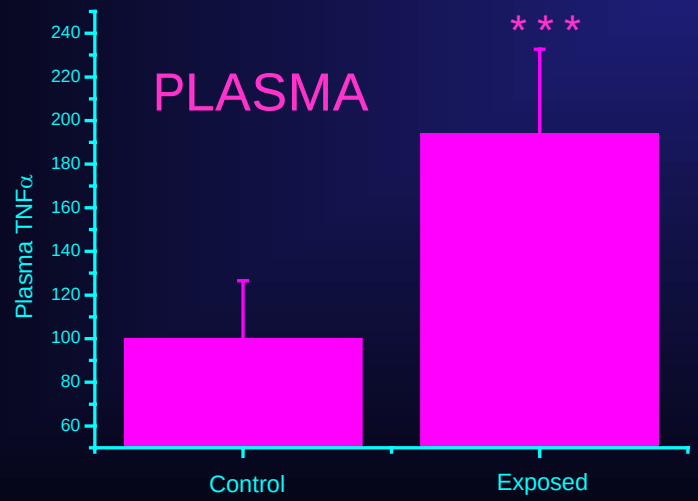
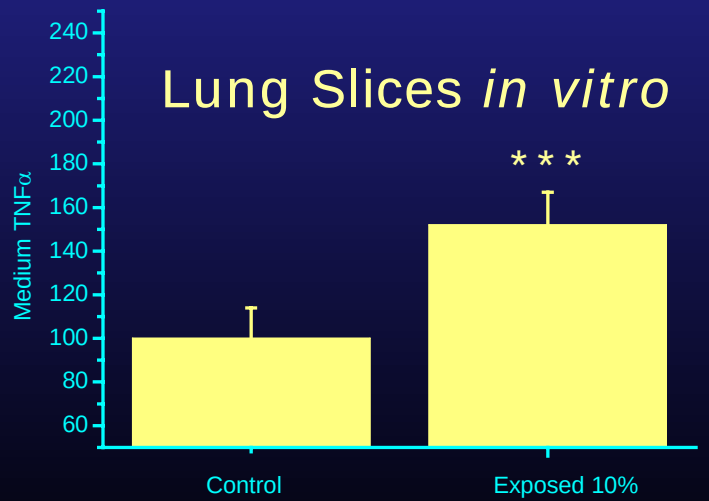
Inflammation : TNFα Production









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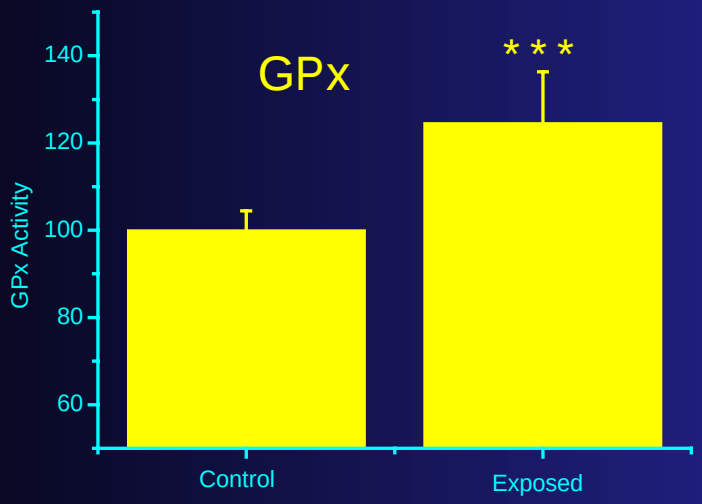
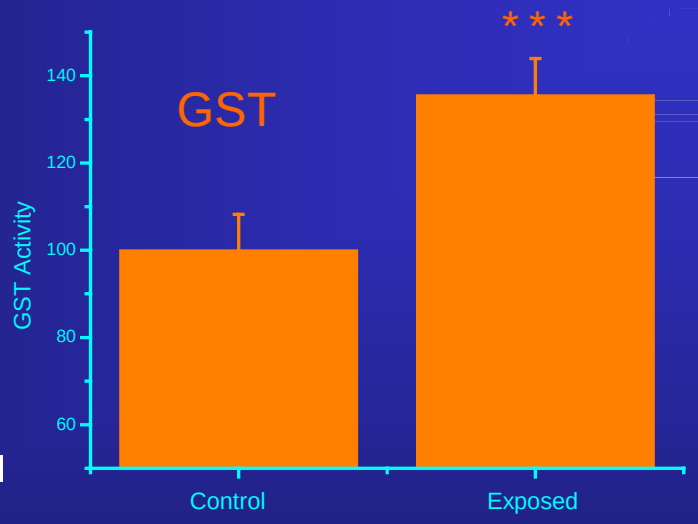
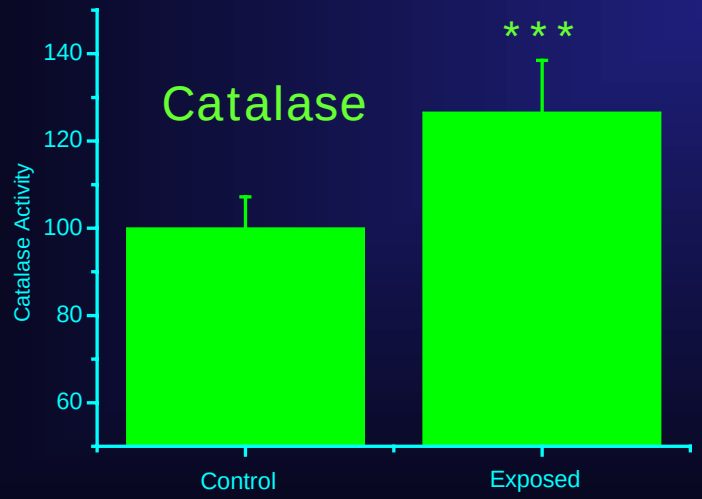
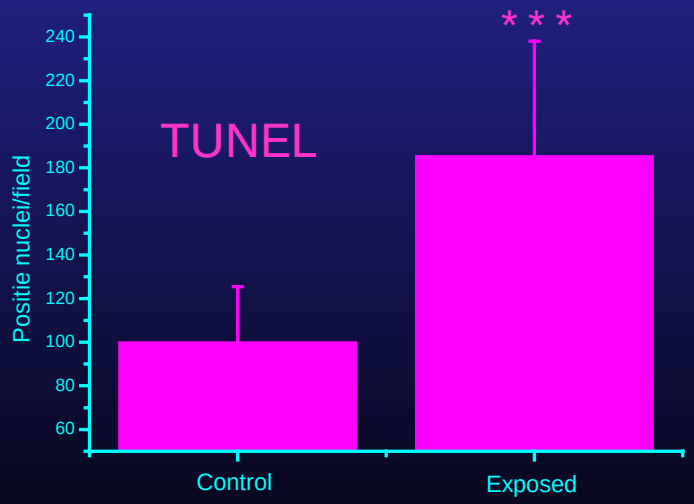
Lung Tissue Investigations









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Lung Tissue Investigations

Excellent correlations between in vitro and in vivo Lung Toxicity patterns with continuous exposure to diluted exhausts

DNA alteration, Inflammation and Oxidant stress

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Experimental Design

Cardiac Investigations

Exposure duration 3hour/day for 2days / week (Exhaust 1/50)

Raw Exhaust Pollutants :

NO 306, NO_2 20, HC 515, Smoke Index 1.29,
PM 3.7 $10^7/cm^3$, 25.8 mg/m^3, mad 106 nm

Normal rats and Chronic heart failure (coronary artery ligation, at least 2 months prior experimentation

Continuous ECG monitoring and Analysis

Heart Rate Variability, QT interval duration,
Search for Arrhythmia episodes

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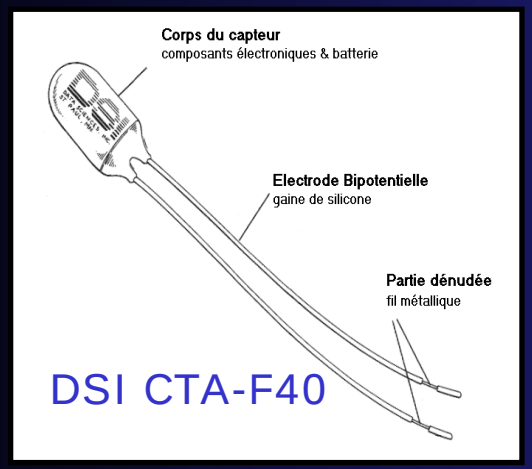
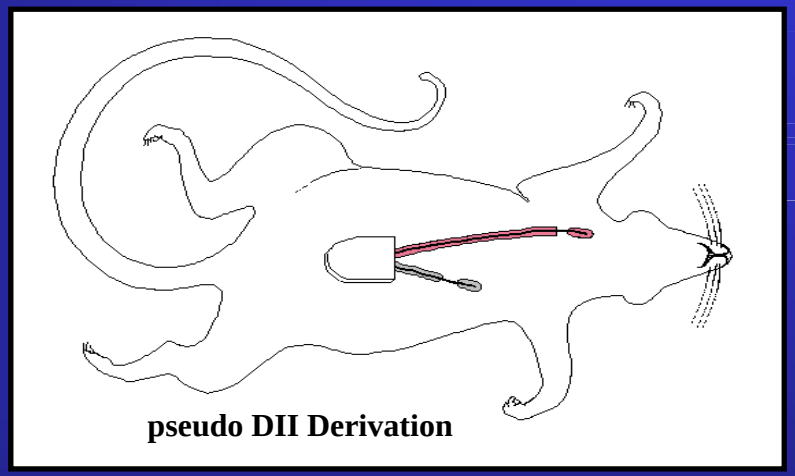
ECG Telemetry

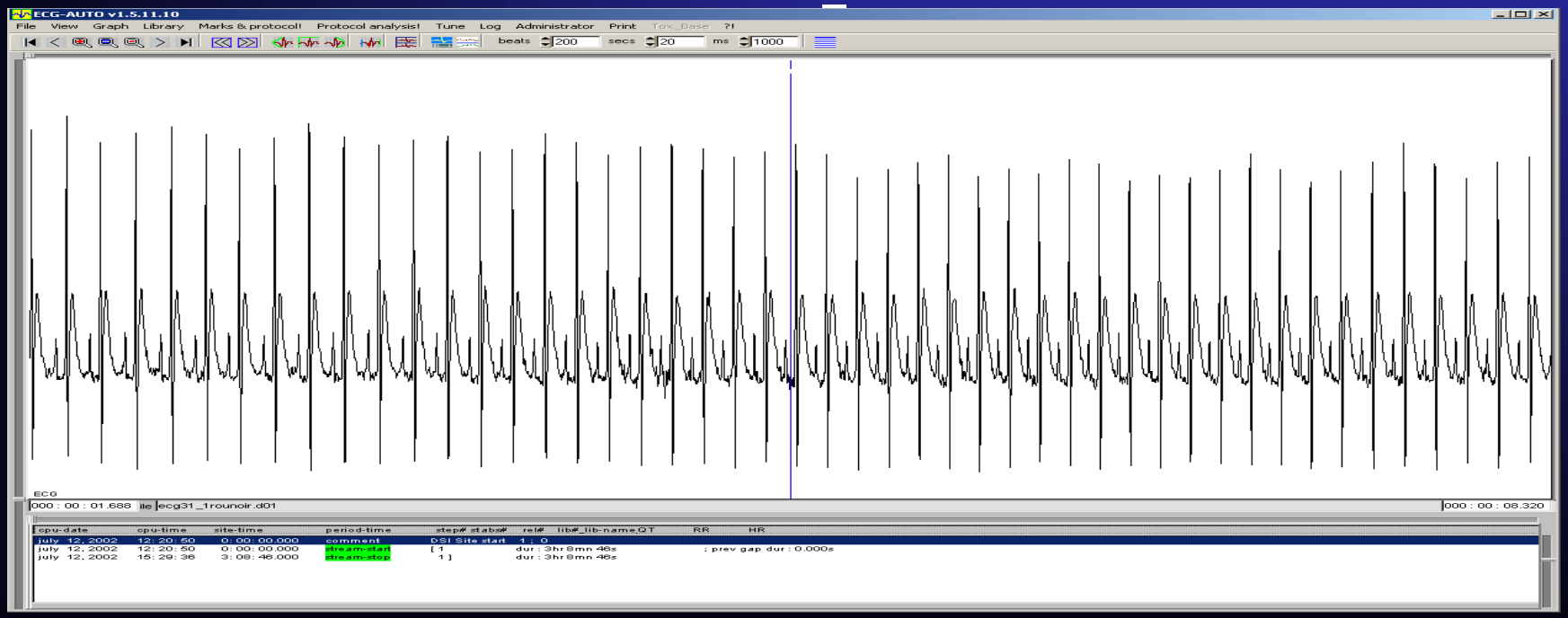


ECG

Temperature

Activity





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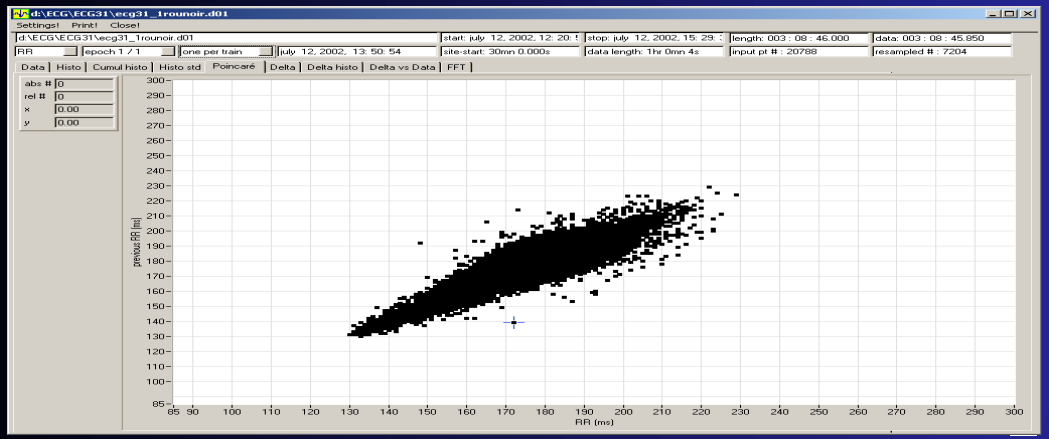
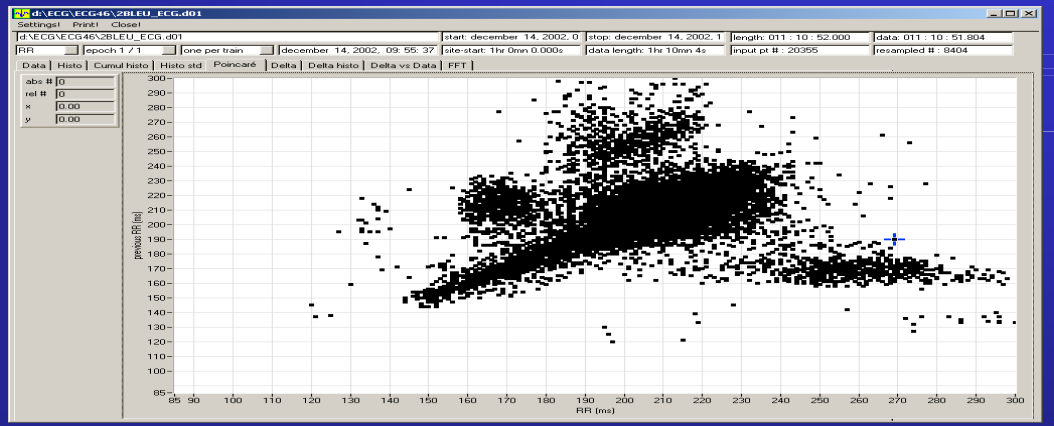
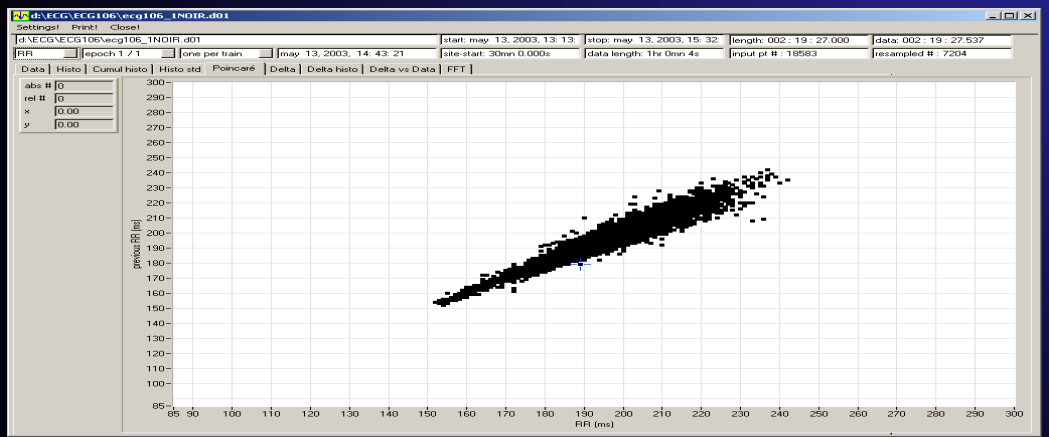
Poincare Plots RR Variability

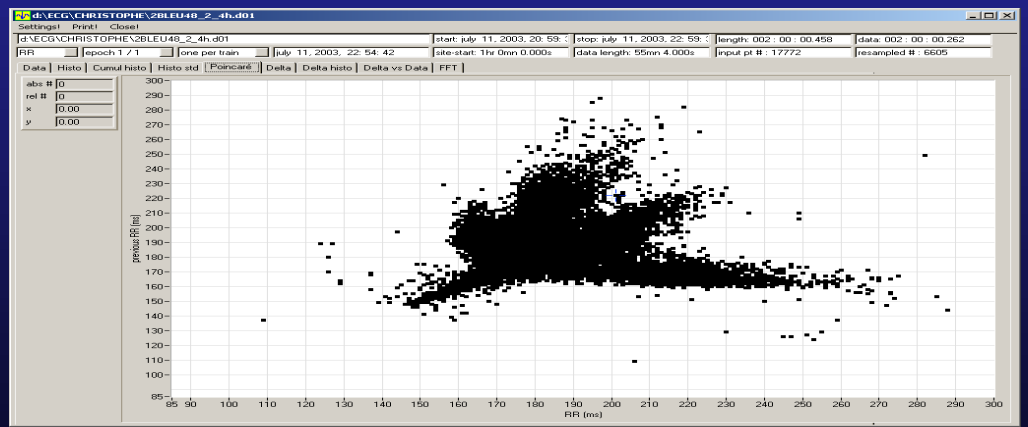
Clean Air





Exhausts 1:50





Sham Rats

MI Rats

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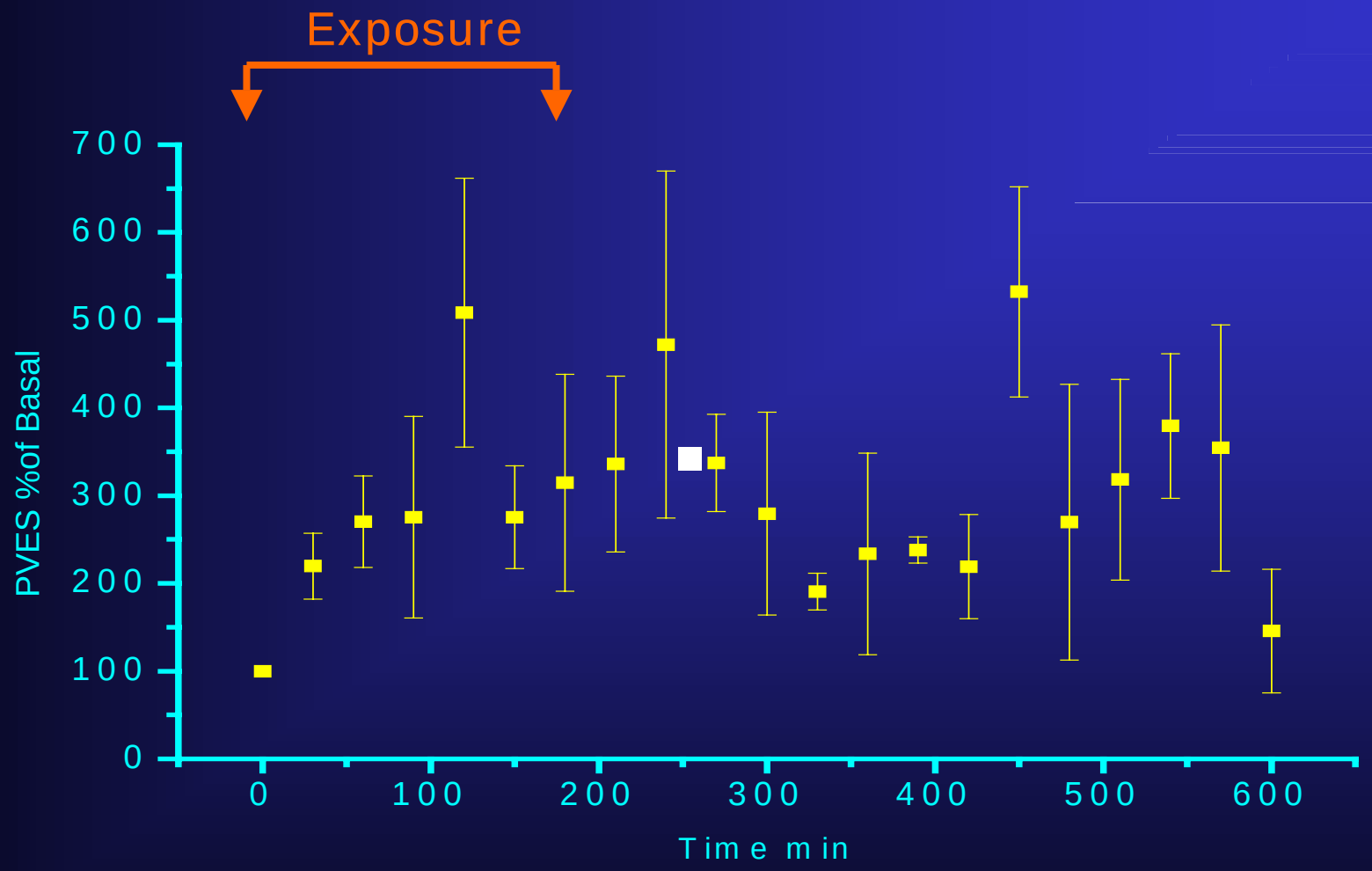
Quantification of PVES occurrence in MI rats Exposed to Exhausts 1/50



Mean of 7 MI rats 4 exposure periods over 2 weeks
Zero time 100% = mean of 60min prior exposure
Each rat serves as its own control

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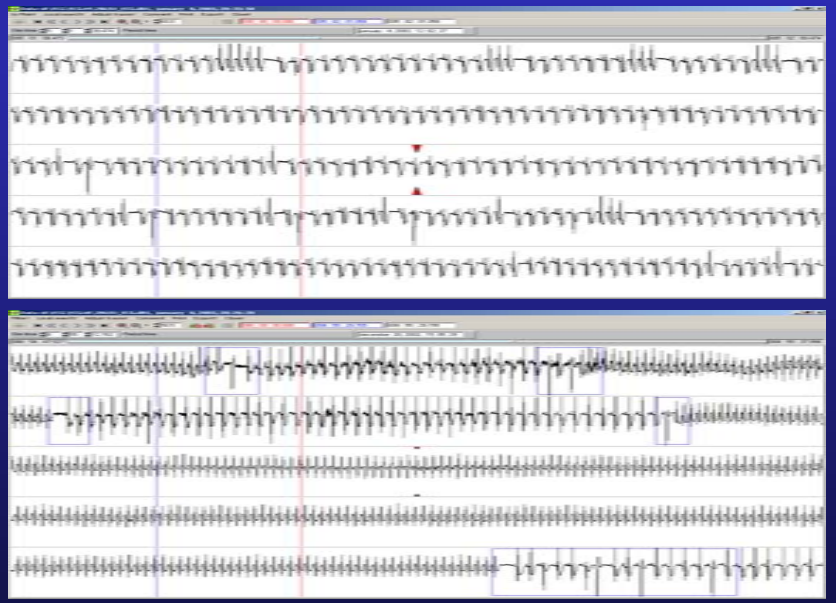
Heart Tissue Investigations

No impact on normal rat ECG frequency and PVES and QT interval
Slight decrease in sinusal HRV

Impact in CHF rats within 15-30 min of exposure
Slight decrease in sinusal HRV
Increased frequency of polymorphous ventricular extrasystoles
Episodes of bigeminy, unsustained and sustained ventricular tachicardia episodes
No impact on QT interval



These data confirm that Chronic Heart Failure may increase susceptibility to pollution episodes

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Conclusion

The Use of experimental designs using continuous flow exposure to complex aerosols for both *in vivo* and *in vitro* experiments allows to better mimick the *in vivo* human situation.

These tools will be most useful to assess the potential improvement of health safety of new fuels, combustion and after-treatment technologies

Help to no regret health based strategies and regulations

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Multidisciplinary Approaches to Airborne Pollutant Health Related Issues

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PORVOO - FINLAND

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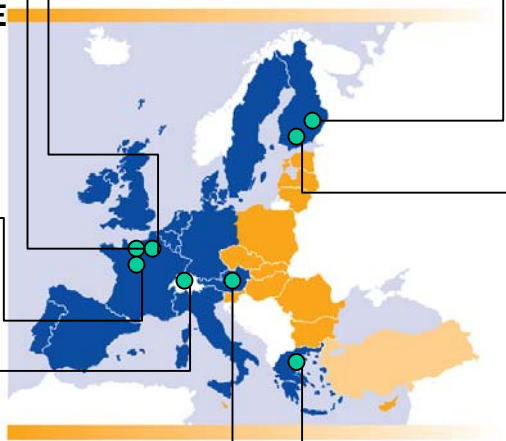

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