Toxicity of Exhaust Gases and Particles from IC-Engines – International Activities Survey (EngToxIn)

1st Information Report for IEA Implementing Agreement AMF, Annex XLII, international activities 2010/2011

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Report :

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*) Abbreviations see at the end of report

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*) Abbreviations see at the end of report

1. ABSTRACT

Exhaust gases from engines, as well as from other combustion – and industrial processes contain different gaseous, semi volatile and solid compounds which are toxic. Some of these compounds are not regarded by the respective legislations; some new substances may appear, due to the progressing technical developments and new systems of exhaust gas aftertreatment.

The toxical effects of exhaust gases as whole aerosols (i.e. all gaseous components together with particle matter and nanoparticles) can be investigated in a global way, by exposing the living cells, or cell cultures to the aerosol, which means a simultaneous superposition of all toxic effects from all active components.
On several places researchers showed, that this method offers more objective results of validation of toxicity, than other methods used up to date. It also enables a relatively quick insight in the toxic effects with consideration of all superimposed influences of the aerosol.

This new methodology can be applied for all kinds of emission sources. It bears potentials of giving new contributions to the present state of knowledge in this domain and can in some cases lead to a change of paradigma.

The present report gives short information about the activities concerning the research on toxicity of exhaust gases from IC-engines in different countries. It also gives some ideas about research of information sources.

It can be stated that there are worldwide a lot of activities concerning health effects. They have different objectives, different approaches and methodologies and rarely the results can be directly compared to each other. Nevertheless there also are some common lines and with appropriate efforts there are possible ways to establish the harmonised biological test procedures.

2. INTRODUCTION

2.1. Actual situation

Emissions legislation is in place in order to control air pollutant emissions of combustion engines. The legislation limits the emissions of the so called regulated components: NO_x, CO, HC and particulate matter. The units are expressed in gram per km or gram per kilowatt-hour. In the near future also a limit of the maximum particulate number emission will be introduced for passenger cars with diesel engine or direct injection gasoline engine (Euro 6, 2013).

When, in the past, more specifically the toxicity of the exhaust gases was needed to be addressed, this was generally done by measuring specific chemical compounds such as PAH (Polycyclic Aromatic Hydrocarbons), BTXE (Benzene, Toluene and Xylen and Ethyl-benzene), aldehydes and 1.3 butadiene.
This has considerable limitations since:
a) the possible combined effect of components may lead to a different toxicity,
b) there may be chemical species which are toxic but which are not measured.

In order to fulfil the need for a more thorough health hazard screening, in recent years biological tests were performed with the exhaust gases (particles and volatiles). These consisted of for example the AMES test as an indicator for the mutagenicity of the compounds; cytotoxicity, as an indicator for cell viability and oxidative potential, as an indicator for the potential to induce oxidative stress. Now that the results of a number of programs are available, the need has arisen to evaluate the quality of the biological screening methods and to come to harmonised (standardised) test methods.

In 2008 the Dutch Ministry of Environment (VROM) has requested the National Institute for Public Health and the Environment (RIVM) to:

a) develop an international network with both engineers and toxicologists/biologists in the area of testing new fuels and engine technologies,
b) coordinate and develop an international harmonized test procedure for toxicity testing of engine emissions.

These activities are done in the framework of the RIVM-VROM project Engine Emission and Health, called SETPOINT. The RIVM has organised 4 international workshops over the past 4 years in which biomedical specialists, toxicologists and engineers from both the private and public sector were brought together. Within these workshops the important biological tests have been discussed including important items as sampling methods (dilution systems), sampling conditions and test cycles.
For evaluation and comparison of the test methods in different international laboratories (both engine and biomedical) the program EngToxNet is defined. The international harmonization with a group of specialists within SETPOINT will be ongoing in parallel and the results of EngToxNet, along with the parallel projects, are needed for further harmonization.

The outcome of the program EngToxNet, namely harmonized test method and data-base with reference data for different engines and fuels, is especially meant to steer future government policies. Currently emission limits on regulated components are becoming more stringent every 3-5 years and billions of Euros are consequently spent to develop the engines that fulfil these requirements. However application of new technologies, new catalysts or fuels might change the chemical composition of the exhaust gas which may reflect a worse quality of exhaust gas with respect to health hazards. With the test method developed in this project, it can be prevented that certain engine or emission control technologies or fuels are introduced which fulfil the requirements but actually form a greater health risks than the old situation.

During the IEA AMF 37th ExCoMeeting in Helsinki, May 2009, it was decided to reinforce the information activities and to help the international collaboration and coordination.

The Swiss and French delegates together with observers from Netherlands organized several meetings and prepared a proposal of an EU-project (per August 2010). As results of these coordinating activities and of the contacts with oversee partners the efforts of coordination and information of the worldwide research on toxicity of exhaust gases from engines with the unified methodology can be summarized with a flow-chart Annex 1 (the mentioned countries are members or observers of AMF).
During the common works it became clear, that the activities have to be divided in several steps and subtasks. As already mentioned the activities on the political-administrative level were called
“SET POINT” and the research projects at technical-scientific level were called EngToxNet (Engine Toxicity Network).

In the proposal of the EU-project participated 9 countries. The search of possibilities of financing this project is still in course. In the meantime there are several national activities and collaborations.

In the present report a special focus on the activities with exposure of human cells cultures or animal tissues to the entire aerosol (combined exposure, whole aerosol exposure) will be given.
The main objective is to make the things comprehensible for non-specialists as far as possible, with no obligation to enter too much into the technical and scientific details.

2.2. Technical and scientific remarks

Kinds of exposure

There are different ways of testing the toxic influences:

a) Epidemiological studies – research on groups of peoples, which were exposed to some notious influences over a longer time. This very work consuming method gives only retrospective information and the results can be cross-influenced by other factors in the research period.
b) Testing on living humans, or animals – "in vivo". Beside the ethical problems, there are tendencies to apply to low dosing for humans and to high dosing for animals. In both cases the observed effects are not realistic and they have to be extrapolated.
c) Testing of biological material in laboratory – "in vitro".
   - Most popular is to collect the toxic material from the emission source, to put it in suspension or in solvent and to expose the cells, cell cultures or tissues (bio-material) to the toxic substances, in liquid phase, independently of the emission source.
     An example is: collecting of exhaust particles, resuspension and testing in submersed cell cultures in vitro. Disadvantages are: no consideration of gas phase and gaseous toxic components, change of particle characteristics and composition during collection and resuspension, no realistic conditions (no air-liquid interface) for the cells from respiratory tract which is the principal way of air pollutants to penetrate into the human body.
   - New method, as mentioned in abstracts, is the combined exposure: exposure to the entire aerosol, (whole aerosol exposure) with all toxic substances acting simultaneously and with realistic repetitive conditions of temperature, humidity, dilution and air-liquid interface.

     In this method the exposed bio-material has to be near to the emission source during the all exposure time.

     In the case of IC-engines, or vehicles the cells are brought to the engine-, or chassis-dynamometer in a specialized vehicles laboratory. Special transportable exposure chambers have been developed for this purpose.

     A highly interdisciplinary collaboration between engine specialists and toxicologists is necessary.

     From both sides: engine as emission source and cell exposure as receiver of pollutants there is a large number of variables, which have to be fixed if a unified methodology should result. These variables are:
     for vehicle: type of engine, operating conditions, type of fuel, lube oil, exhaust gas aftertreatment, diverse technical modifications;
     for exposure: bio-material (cells, tissues), exposure conditions (temperature, humidity, dilution), exposure time, incubation, repetitions.

     The exposed biological material can be:
     - cell monocultures – focusing on one cell type, no cellular interplay
     - multicellcultures (e.g. human airway triple cells model) – more advantageous,
     - animal lung tissue – extrapolation from animal tissue to humans.

In several conferences (see activities SET POINT & EngToxNet, pt. 2.1.) the conditions of combined exposure were discussed and most of them were accorded in the meeting at ADEME, Sophia Antipolis (Nizza), Oct. 16th, 2009. It was accorded to continue the works on the common methodology with multicellcultures and with animal lung tissues.

Toxicological tests – endpoints

The toxicological tests can be divided in following groups:
- cell viability and genotoxicity – regarding cells modifications and mortality (number of dead cells),
- oxidative stress,
- inflammatory reactions.

The tests mentioned by project partners are given in annex A2. Most of the tests are normalized.

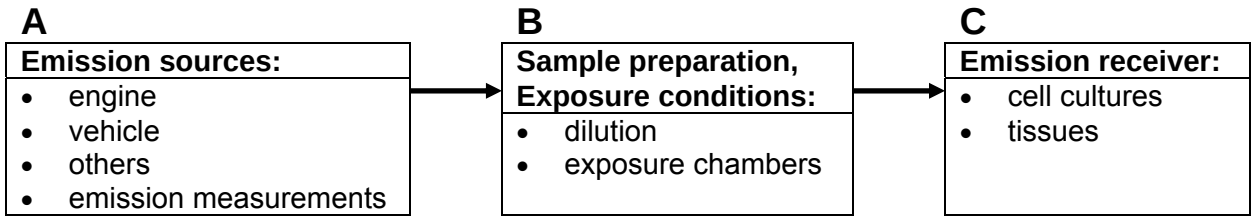
In free research the scientists may modify, or create other testing methods, according to different points of view and different objectives.
The efforts to establish a harmonized procedure nevertheless consider only standard tests.

A well known standard test of mutagenicity on salmonella bacteria is the AMES-test. This test can be attached to each research activities, but the toxicologists working with combined exposure do not consider it as representative for human cells and do not recommend it for the harmonized procedures.

Some further descriptions of biological processes and test methodologies are given in annex A3.

2.3. Interdisciplinarity & complexity

The new exposure method of cells to the entire aerosol, which is described in pt. 2.2. (c) (aerosol exposure) can be graphically represented as in following chart:



Interdisciplinarity

Part A is performed by a laboratory, which can measure the emissions of engines, or vehicles according to the legal methods. This requires certain complexity of installations and measuring systems and a specialization of the participating personnel.
Emission measurements i.e. physico-chemical characterization concerns both: the legally limited and unlimited gaseous and particulate components.
Usually the limited components (CO, HC, NO_x, particle mass & counts) are analysed as standard by the legally measuring laboratories. The analytics of other unlimited components, like differential HC including PAH, nitric compounds or traces of substances is performed in collaboration with specialists for organic, or unorganic analytics.

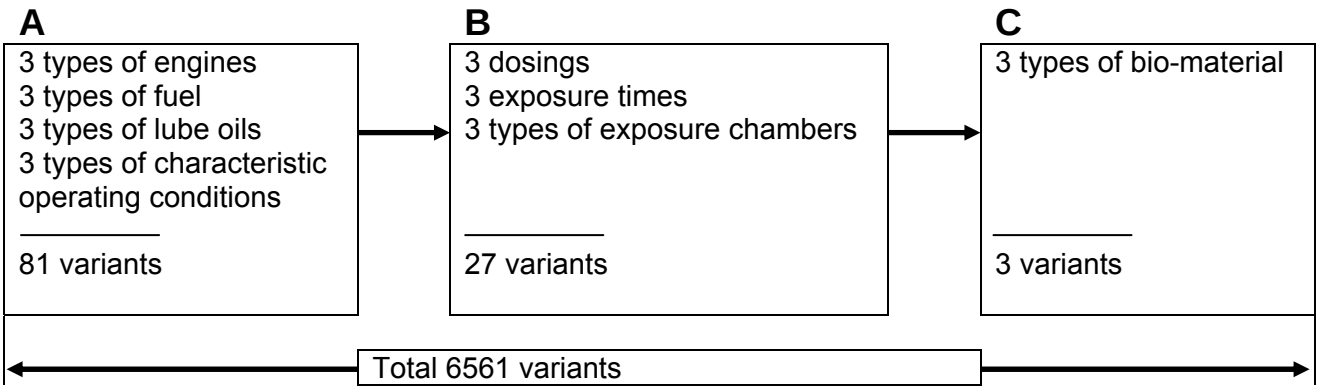
Part B – the conditioning of emission sampling – is prepared by specialists of measuring technics and control. It can be handled by the technicians from Part A.

Part C – the preparation, transport, exposure, incubation and toxicological tests are performed by toxicologists.

These remarks show that the research with combined aerosol exposure is a highly indisciplinary activity.

Complexity

The complexity of the investigated matter can be depicted by a simple example with a very modest supposition of 3 variants of some variables:



It is clearly to see that for a harmonized procedure certain variables have to be fixed.

Which combinations of emission sources and biological materials have to be investigated with preference?
The search for the "right way" is similar, like the "design of experiment DoE" for optimizing of systems with high number of variables.
In toxicological research the desired combinations of test variants are usually set by the experience of the working specialists (see: meetings and conferences of SETPOINT & EngToxNet).

In addition to the objective complexity of the investigated matter, the other complexities of analytical procedures and of organization can be mentioned.

2.4. How shall we call the research?

The research on emissions (part A) can be called "physico-chemical characterization" of exhaust gases.

In some research programs with profound analytics of nonlegislated components the toxicity research is mentioned. This is not right in strict meaning, since there was no research about biological responses of exposed bio-material.

There are officially used methods of describing the toxic potential of mixture of substances, like EPA toxicity equivalence TEQ.

These methods assume a linear dependence of toxic effects and amount of toxic substance and they neglect the possibilities of non linear influences and of other possible effects connected with the simultaneous interaction of different substances (multiple effects, superposition of effects, cross influences).

This supposition of proportionality between the concentration of toxic substance and the toxic effects (dose-response) is surely sufficient in most simple cases. But it is not satisfactory for special applications with many complex pollutants.

This opinion is supported by many biologists and toxicologists and it is the reason for proposing new universal exposure methods.

If we accept that the relationship: toxic substance – toxic effects is not always known, we should make difference in terminology between the research on the emission source and the research on the bio-material.

The authors propose to use following convention:

The research on emission source:
- physico-chemical characterization of emissions, or
- investigation of toxic potentials of the emission source.

The research on living cells: (epidemiological, in vivo, in vitro):
- research on toxicity.

This terminology will be considered in the present report.

3. ACTIVITIES

The information obtained from several partners is shortly commented and the received information notes and reports, as well as some positions from literature are given as annexes of the report.

3.1. France

(contact: jean-paul.morin@univ-rouen.fr)

Important developments of the biological exposure to the complex aerosol (aerosol exposure) were initiated and performed in the French network.
The exposure systems for animal lung slices and for cell cultures were developed and tested with the Greek partners, [1, 2] and applied for toxicity research on engines and vehicles (example [3]).
Actually the French network starts a project MAETAC (Méthodes Alternatives pour l'Evaluation de la Toxicité des Aérosoles Complexes), which will compare the results of exposures: on line (whole aerosol), off line (resuspended PM) and mutagenicity.

Information from Dr. J.-P. Morin and title pages of the mentioned references see annex 4.)

Advantages of whole aerosol exposure system are:
- no alteration of both gaseous phase and PM physicochemical properties,
- interactions of aerosol and biological sample simulating the real "in vivo" situation (sedimentation and diffusion),
- no alteration of pollutant bioavailability,
- global approach of exhaust impact.

3.2. Netherlands

(contact: ruud.verbeck@tno.nl; ingeborg.kooter@tno.nl)

Information from TNO:

Earlier work at TNO published this year (2011):
- Toxicological characterization (cytotoxicity and mutagenicity via Ames test) of diesel engine emissions (DAF XE355 Euro III truck engine) is using biodiesel and a closed soot filter [4] (some fragments see annex A5).

2011 R&D at TNO:
- Effects of exposure of diesel exhaust emissions (Euro III truck engine) on human alveolar epithelial cells (A549) using a Cultex exposure unit (whole aerosol exposure).

2012: work that will start in 2012:
- Effects of exposure of diesel exhaust emission on bronchial epithelial cells from COPD and asthma patients.

TNO (Netherlands National Laboratories www.tno.nl) have excellent possibilities of interdisciplinary collaboration of engine specialists with toxicologist. TNO also collaborates with RIVM (Netherlands Institute of Environment and Public Health www.rivm.nl).
In [4] TNO uses the PM-extract exposure, but it stresses the necessity of international harmonization and validation of bio-toxicological test methods, see annex A5-2.

Activities National Institute for Public Health and the Environment (RIVM),
Contact: Miriam E. Gerlofs-Nijland, Centre for Environmental Health (MGO)
miriam.gerlofs@rivm.nl; flemming.cassee@rivm.nl

- Continuation of the network SETPOINT (Screening Emissions for Toxic Potential - Organising INTernational harmonisation) which promotes knowledge transfer and harmonization of hazard screening of engine emissions and the critical evaluation of these developments to guide policymakers and regulators. A COST Action will be set up for funding network activities;
- Publish the international and harmonised draft health screening approach agreed on during a number of workshop in 2008-2009 coordinated by the Dutch National Institute for Public Health and the Environment RIVM;
- Project "Engine emission and Health" funded by the Dutch Ministry of Infrastructure and the Environment to keep up with professional literature and support policy with ad hoc questions regarding traffic-related health effects;
- Toxicity testing of engine emissions (in vivo animal/human) in collaboration with divers partners;
- Participation in the role as advisor in the Engine Toxicity Network (EngToxNet) which has been formed with the aim to launch an international (probably European) project for validation of the harmonised test methods for toxicity screening of engine emissions.

3.3. Switzerland

(contact: jan.czerwinski@bfh.ch)

Different activities and collaborations in Switzerland are represented in annex A6.
In both domains: physico-chemical characterization of the pollutants and bio-toxicological responses there are several deeply specialized institutes.
The question of nanoparticles (NP) and health effects was early recognized and investigated on several places. In the domain of NP-measurements Swiss NP-Network contributed a lot to the PMP-Program of the ECE GRPE. In the domain of health effects the yearly organized Nanoparticle Conference at the Federal Institute of Technology (ETH Zürich) contributed very much to the interdisciplinary knowledge exchange between toxicologists and engineers
(www.nanoparticles.ethz.ch, see chap. 4.1.).

In several studies it was shown, that there is an increased penetration of ultrafine particles into the cells and there are dose-dependent effects on the cells function. The biological responses depend also on the type of cells used for the investigations. As examples the title pages of the studies [5] & [6] are given in annex A7.

The activities with whole aerosol exposure started 2007 with the research on 2-stroke scooters, which was ended with the Ph.D. Thesis [7]. This PhD. Thesis is available on the AFHB homepage at: www.afhb.bfh.ch → official reports → Dissertation L. Müller.

The toxicological research of exhaust aerosols from Diesel passenger car is continued in the Swiss Network with the project "BioToxDi" (Biofuels, Toxicity, Diesel), see annex A8. In this project the principal influences of the emission source, like different fuels, lube oils, aftertreatment, etc. on the biological responses of a triple cell cultures are investigated.

As conclusions up to date it can be remarked, that:
- there is a clear influence of exhaust gas quality on the cytotoxicity, oxidative stress and inflammatory reactions of cells,
- the exposure of cells to the combined aerosol (with gaseous and particulate toxic components) is a very useful method of research of toxicity; it is proposed to apply this method for all kind of pollution sources.

3.4. Germany

(contact: jan.knebel@item.fraunhofer.de)

At the Fraunhofer Institute for Toxicology and Experimental Medicine in Hannover there are large experiences with different bio-toxicological test methods, also with the whole aerosol exposure. Own exposure system was developed. For more information see the letter and the reference [8] in annex A9.

At the Fritz Haber Institute of the Max Planck Society in Berlin a study of exposing human blood cells to the Diesel soot in suspension was performed, [9], annex A10.

Many activities concerning the detailed physico-chemical characterisation and mutagenicity of engine exhaust gases are known from the network FJRG (Fuel Joint Research Group):
- Institute of Agricultural Technology and Biosystems Engineering,
  Johann Heinrich von Thünen Institute, Braunschweig, Germany
  (contact: axel.munack@fal.de),
- Coburg University of Applied Sciences, Coburg, Germany
  (contact: juergen.krahl@hs-coburg.de),
- Steinbeis Transfer Center for Biofuels and Environmental Measurement Technology, Coburg, Germany
  (Contact: buenger@bgfa.de).

Mutagenicity of particle mass from RME was investigated by this network in [10]. It was found that the mutagenous activity with biodiesel is much higher than with base fuel and it is the highest with B20, (nonlinear dependence on biofuel ratio).
In opposite to that another German research group found the mutagenicity of biocomponents (rapeseed oil) much lower, [11].

Annex A11 represents information from FJRG and the summary of a study concerning the legislated and non legislated emissions and mutagenicity (Ames Test) with different fuels on HD Diesel engine, [12]. This study is a task-sharing contribution of the German partners to the IEA AMF Annex XXXVII (Fuels & Technology Alternatives for Busses).

(contact: R. Winkelmann@fnr.de)

FNR (Agency for Renewable Resources) commissioned a study to assess the emissions of biofuels combustion in different types of engines. This study was conducted by two research groups

1. Munack, Krahl, Bünger (see Fuel Joint Research Group)

2. Dr. Jürgen Blassnegger (Institute for Internal Combustion Engines and Thermodynamics, Graz University of Technology)

   Markus Knauer (Technical University of Vienna, Institute for Internal Combustion Engines)

   Prof. Karl-Werner Schramm (Technical University of Munich, Ecotoxicology - http://www.wzw.tum.de/~schramm/; Helmholtz-Zentrum München; institute of ecological chemistry; http://www.helmholtz-muenchen.de/en/home/index.html?fontSize=A)

   Prof. Reinhard Nießner (Technical University of Munich, Institute of Hydrochemistry IWC; http://www.ws.chemie.tu-muenchen.de/)

   Manfred Wörgetter (FJ BLT Wieselburg)

Investigation of mutagenic effects (Ames test, EROD-Assay) was part of the study. Both research groups developed and used their own sampling methods. It has been found that, all in all, the mutagenous activity with biofuels and fuel blends is not significantly higher than with base fuels. However, it has also been found that sampling methods may affect the results. Thus, in an ongoing follow-up project both methods are to be verified.

3.5. Denmark

(contacts: j.bonlokke@dadlnet.dk; jb@mil.au.dk; Steffen Loft: stl@sund.ku.dk)

There is collaboration between the universities of Aarhus (au) and Copenhagen (ku) and Danish Technological Institute. There are many activities and competences and air-liquid exposure of cells on an engine emission facility is in preparation with a partner University of Lund.
Short information from University of Copenhagen is given in annex 12.

3.6. Norway

(contacts: per.schwarze@fhi.no; otto.andersen@vestforsk.no)

Several activities and international collaborations of the Norwegian Institute of Public Health are mentioned in annex A13. There are among others extensive experiences with exposure of different cells types to the “extracted and fractionated” organic material.

The Western Norway Research Institute (WNRI) is active in international projects on bio-fuels and toxic potentials. WNRI is specialized in molecular dynamics simulations which allow investigating the interactions of nanoaerosols and chemicals with the cells. A short information report of WNRI is given in annex A14.

3.7. Czech Republic

(contacts: michal.vojtisek@tul.cz; jtopinka@biomed.cas.cz)

There is an intense interdisciplinary collaboration between the Department of Vehicles and Engines, Technical University of Liberec and the Institute of Experimental Medicine of the Czech Academy of Science. There is participation on the activities of harmonization the methodology of risk assessment RIVM & EngToxNet. Further information, see annex A15.

3.8. Finland

(contacts: Maija-Riita-Hirvonen@uef.fi; jorma.jokiniemi@vtt.fi)

At the University of Eastern Finland (UEF) there are activities with air-liquid exposure of cells to the whole emission aerosol. There is collaboration with the National Research Laboratories VTT. Information see annex A16. Important to mention is the research with aged aerosols, so called secondary organic aerosols (SOA), which occur in the real world exposure. The ageing of aerosol for research is conducted in special ageing chambers using UV light radiation of controlled intensity.

3.9. Greece

(contacts: A.G. Konstandopoulos: agk.@cperi.certh.gr)

The Aerosol & Particle Technology Laboratory (APTL) of the CERTH / CPERI has a long tradition in research on nanoaerosols from engines. There also is high competence of physico-chemical characterization, see information, annex A17. For toxicological research there is a collaboration with the Department of Biology of the Aristotle University.
APTL participated on an EU-project MAAPHRI (see A17-2), developed the exposure chambers and collaborated actively with the French Network (see chap. 3.1.)

3.10. USA

(contacts: sioutas@usc.edu; ayala@arb.ca.gov; maddenmichael@epa.gov)

There are many activities concerning air pollution, traffic emissions and health effects in California, which is regarded as a birthplace of the exhaust emissions legislation in the 60-ties and 70-ties.
Except of the Californian Air Resources Board (CARB) there are other known institutions supporting the research of academia. Some of them are:

- South Coast Air Quality Management District (SCAQMD),
- Southern California Airborne Particulate Matter Center (SCAPMC),
- Asthma Allergic Disease Research Center (AADRC).

Extensive information about running projects is given in annex A18 from Prof. Constantinos Sioutas, University of Southern California. There are different topics of research: from physico-chemical characterization and distribution of pollutants, to exposures and effects on different target groups of people.
As examples of research results about health effects of particulate pollutants fragments of references [13] & [14] are given in annex A19.
Generally there are no doubts about the penetration of nanoparticles into the human organism and about the negative acute or potential health impacts. The last ones depend on many factors, like: composition of nanomaterial, target organs (or cells), dosing (i.e. exposure time & concentration). There are many variables and each project, like usually in the research, can open new questions.

Information and references from EPA, Chapel Hill, North Carolina are given in annex A20. There is a close collaboration with the University of North Carolina School of Public Health (http://www.sph.unc.edu).
In both institutions there are competences for the research with all kinds of exposure.

There also are extensive activities of the Health Effects Institute (HEI … www.healtheffects.org).
Th. W. Hesterberg from Navistar reported at the Nanoparticle Conference in Zürich, June 2011 (see annex A23) about some projects with industry:

1. Advanced Collaborative Emissions Study (ACES)
    - Managed by the Health Effects Institute
    - Funded by government agencies and industry
    - Liefespan inhalation study in rodents
    - Lung disease and cancer are main endpoints
    - Two more years to complete

2. Diesel Exhaust Lung Cancer Studies
    - Human workplace studies show small increase in lung cancer, but no exposure-response demonstrated – Same small increase seen before dieselization of trucks
    - Miners, who have highest DE exposures show no increase in lung cancer
    - Lung cancer not found in mice or hamsters and only at very high “lung overload” exposures in rats

- Thus, there is little evidence that DE causes lung cancer at occupational or environmental exposures
  Hesterberg et al. Critical Rewiews in Toxicology 36:727-726, 2006

3. Diesel Exhaust Human Volunteer Studies
   - High Diesel exhaust nanoparticles exposures may elicit transient, subclinical effects in human volunteers
   - Effects generally less or not seen at lower exposure levels
   - Responses similar to those observed with larger particles
   - Effects not observed with New Technology Diesel Exhaust
   - These studies do not provide evidence of a unique toxicity of nanoparticles compared to larger particles
     Hesterberg et al. Inhalation Toxicology 22(8):679-694, 2010

3.11. Canada

(contacts: subramanian.karthikeyan@hc-sc.gc.ca, paul.white@hc-sc.gc.ca)

Annex A21 contains the short information from the highly specialized laboratories of the National Research Council, Canada.
There are works and experiences with different types of exposure with the objectives to attain the most realistic exposure route.

4. Other Information Sources

4.1. Literature

There is a huge amount of literature.
As already pointed out in the chap. 2.4., there is not always a clear differentiation of notions between physico-chemical characterization and toxicological research. Several studies dealing with profound analytics of pollutants composition attach to the study some toxicological elements like Ames Tests, or toxicity equivalence TEQ. This type of studies is usually done by engine specialists together with chemical analysts.

Examples physico-chemical characterization

Some examples of research on toxicological potentials (physico-chemical characterization) are given in [15, 16 & 17], title pages see annex A22.
In [15], the results of a big US-project ACES (Advanced Collaboration Emissions Study) are described. In this project with participation and support of DOE, EPA, CARB, HEI, EMA and others 795 unregulated engine exhaust emissions species were characterized on 4 makes of new generation HD Diesel Engines. To mention are traces of Dioxines & Furanes, PAH & Nitro PAH and metals.

In [16] the analysis of engine lube oil is represented. This is an important topic during the operation of engines with bio-fuels, since the biocomponents cause a higher oil dilution and quicker oil quality degradation.
On the other hand in the low-emitting modern engines there is a higher share of particulate emission originating from lube oil. The lube oil chemistry and additive packages cannot be neglected by the consideration of toxic potentials.

In [17] emission characterization of light-duty vehicles together with a simplified bioassay test (Microtox) were performed. Some doubts about the correlations of bio-results and PM chemical speciation were expressed. The high efficiency of Diesel particle filter in eliminating the particles and minimizing the toxic potentials were confirmed.

Bigger literature lists & overviews

1. The interdisciplinary information exchange is promoted since many years at the Nanoparticle Conference organized yearly at the Federal Institute of Technology ETH Zürich (www.nanoparticles .ethz.ch). Annex A23 shows the presentations from last 3 years 2009, 2010 & 2011. Further information can be asked and a CD can be ordered at: ttm.a.mayer@bluewin.ch
2. From the German Network (chap. 3.4) working with engine emissions, health hazards and workplace protection there is an overview of the current knowledge [18] with 70 literature positions (useful fragments of texts see annex A24).
3. In a review article from the New York University School of Medicine [19] (text fragments, see annex A25) the complexity of real world exposure and the necessity to approach as much as possible this real exposure are underlined. There are approximately 200 literature positions at the end of the article (49 pages).
4. Lists of bibliography from DOE about vehicle technology, health impacts and hazards are given in separate attachments annexes A26 (100 positions) & A27 (87 positions).
   (contact: kevin.stork@ee.doe.gov … US delegate to IEA AMF).
5. Bibliography of VERT Association concerning the health effects is given in annex A28
   (321 positions). (contact: ttm.a.mayer@bluewin.ch; VERT … Verification of Emission Reduction Technologies see: www.vert-dpf.eu; www.vert-certification.eu)
6. 3 lists of bibliography from University of Rouen, F are given in annex A29. There are main topics: human exposure, aerosol exposures to engine exhaust, cigarette smoke.

4.2. Internet

There are vast possibilities of information research on internet.
We want to mention the homepages of the concerned institutions and universities and the Wikipedia addresses:
http://wikipedia.org//wiki/toxicity
http://en.wikipedia.org/wiki/Exhaust_gas
http://wikipedia.org//wiki/Diesel_exhaust

To mention is the publication page of the Health Effects Institute (HEI):
http://pubs.healtheffects.org

5. CONCLUSIONS

- The research activities about toxicology have a large extend in several countries and focus on different pollution sources.
- The principal methods of research are:
  - epidemiological or group-related studies,
  - exposures in vivo (humans or animals)
  - exposures in vitro (different kinds of supply of pollutant, different exposed bio-material).
- The methodology of whole aerosol exposure (gaseous & particulate compounds) is still not very wide spreaded – as major limits the necessity of a highly interdisciplinary approach and high personal / material efforts can be regarded.
- Nevertheless, this methodology offers the best balance between the objectivity of the biological response and the time-to-results.
- A lot of work was done to pave the way of this method to become an international standard – further efforts are necessary.

- An important point in the discussions is to make difference between the research on physico-chemical characterization of the pollution source of gas (tox-potentials) and the toxicity research (bio-responses, tox-effects).
- The details of methodology of research are often not clearly to see from the publications, but there are several countries already working with the newest method of whole aerosol exposure and the other countries have excellent potentials to do it.
- The establishment of an harmonized international biological test method is possible.

6. ACKNOWLEDGEMENTS

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8. ABBREVIATIONS

ADEME	Agence de l'Environnement et de la Maîtrise de l'Energie, France
AECC	Association for Emission Control by Catalyst (www.aecc.be)
AFHB	Abgasprüfstelle der Fachhochschule, Biel CH, (www.afhb.bfh.ch) (Lab.For Exhaust Gas Control, Univ. of Appl. Sciences, Biel-Bienne, Switzerland)
AMF	Advanced Motor Fuels
BfE	Bundesamt für Energie, CH (SFOE)
BAT	best available technology
BAFU	Bundesamt für Umwelt, (Swiss EPA, FOEN)
CARB	Californian Air Resources Board
CERTAM	Centre d'Etudes et de Recherche Technologique en Aérothermique et Moteur
CERTH	Center of Research & Technology Hellas

COPD	chronic obstructive pulmonary disease
CPC	condensation particle counter
CPERI	Chemical Process Engineering Research Institute
CVS	constant volume sampling
DMA	differential mobility analyser
DOE	US Department of Energy
DPF	Diesel Particle Filter
EMA	Engines Manufacturers Association (US)
EMPA	Eidgenössische Materialprüfungs- und Forschungsanstalt
EngToxNet	Engine Toxicity Network
EPA	Environmental Protection Agency
ETHZ	Eidgenössische Technische Hochschule Zürich
EV	Erdöl Vereinigung, CH (www.swissoil.ch)
FJRG	Fuel Joint Research Group, D
FNR	Fachagentur Nachwachsender Rohstoffe, D
FOEN	Federal Office of Environment (BAFU)
GRPE	Groupe Rapporteur Pollution et Energie
HEI	Health Effects Institute
IA	Implementing Agreement
IEA	International Energy Agency
INSERM	Institut National de la Santé et de la Recherche Médicale, F
INSOF	insoluble fraction
JRC	EU Joint Research Center, Ispra It.
NP	nanoparticulates
PAH	polycyclic aromatic hydrocarbons
PM	particulate matter, particulate mass
PMP	Particle Measuring Program of the UNO ECE GRPE
PN	particles number
PSI	Paul Scherrer Institut, Switzerland
RIVM	NL National Institute of Public Health
SAE	Society of Automotive Engineering (www.sae.org)
SAG	Swiss Aerosol Group (medical)
SMPS	scanning mobility particles sizer
SOA	Secondary Organic Aerosol
SOF	soluble organic fractions
SWRI	South West Research Institute
TEF	Toxicity Equivalence Factor
TEQ	Toxicity Equivalence TEQ = sum (TEF_i x $concentration_i$)
TNO	NL National Research Laboratories
TPN	total particle number
TTM	Technik Thermische Maschinen, Niederrohrdorf, CH
VROM	NL Ministry of Environment
VSS	Verband der Schweizerischen Schmierstoffindustrie (www.vss-lubes.ch)
VTT	Technical Research Center of Finland

9. ANNEXES

A 1 Efforts of coordination and information of the worldwide research on toxicity of exhaust gases from engines with unified methodology of aerosol exposure

A 2 Toxicological tests - endpoints

A 3 Introduction in test methodologies and some biological processes

A 4 Information & references, France

A 5 Reference from TNO, Netherlands

A 6 Activities & collaborations in Switzerland

A 7 References: Nanoparticles & Health Effects, Switzerland

A 8 Toxicity of Diesel Exhaust, project BioToxDi, Switzerland

A 9 Information & reference from Fraunhofer Institute, Germany

A 10 Reference from Max Planck Society, Germany

A 11 Information & reference from network FJRG "Braunschweig-Coburg", Germany

A 12 Information from University of Copenhagen, Denmark

A 13 Information from Institute of Public Health, Norway

A 14 Information from West Norway Research Institute (WNRI)

A 15 Information Czech Republic

A 16 Information from University of Eastern Finland

A 17 Information from Greece

A 18 Information from University of Southern California, LA, USA

A 19 References about particulates & health effects, USA

A 20 Information from EPA, Chapel Hill, NC, USA

A 21 Information from Health Canada

A 22 Examples of physico-chemical characterisation [15, 16, 17]

A 23 Health Sessions NPC Zürich 2009, 2011, 2011

A 24 Knowledge overview Ruhr-University Bochum (IPA), [18]

A 25 Review Article of New York University, [19]

Electronic separate data

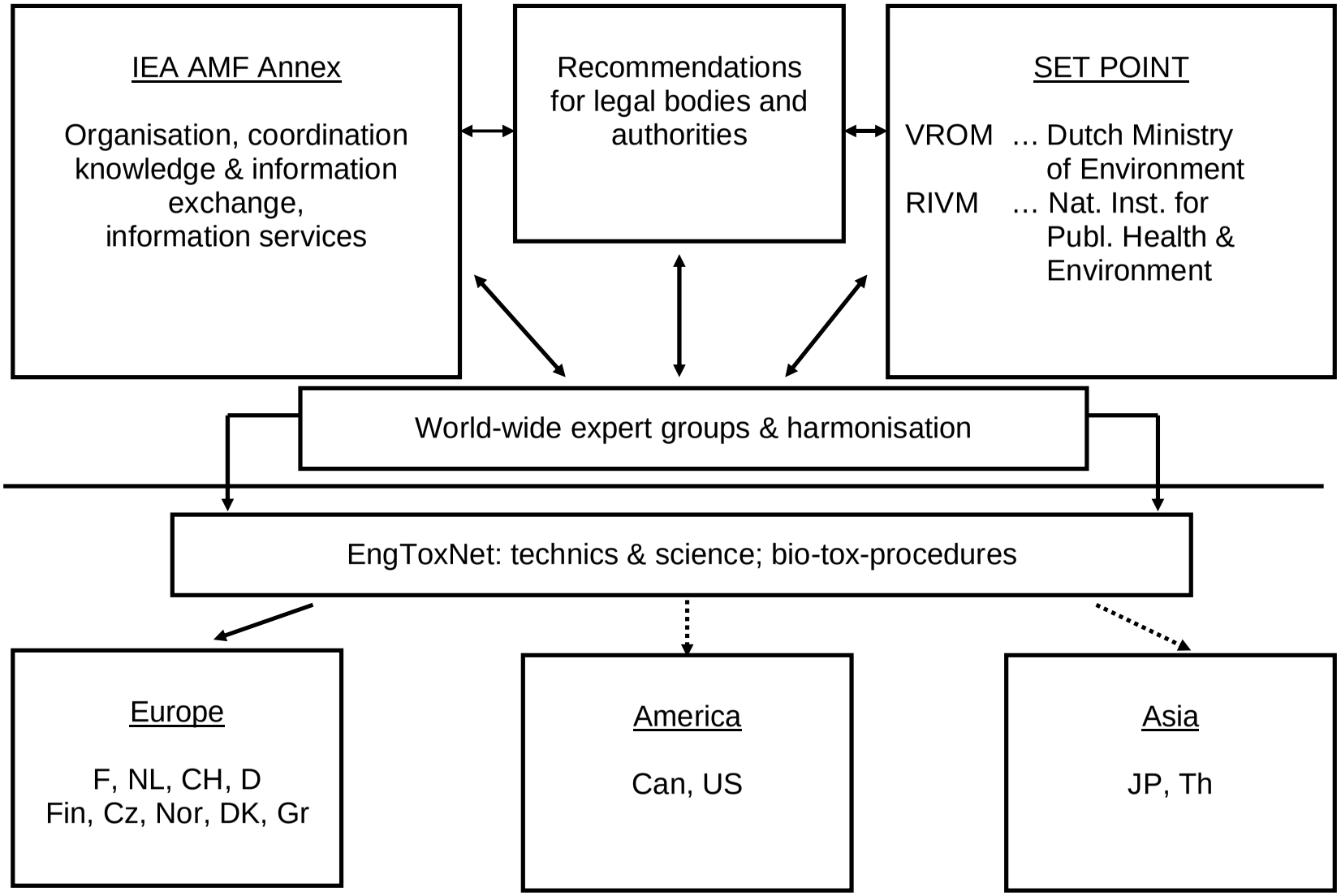
A 26 Bibliography DOE (US) Vehicle Technologies & Health Impact

A 27 Bibliography DOE (US) Health Hazards

A 28 Bibliography Health Effects of the VERT Association

A 29 Bibliography from University of Rouen

Efforts of Coordination and Information of the Worldwide Research on Toxicity of Exhaust Gases from Engines with Unified Methodology of Aerosol Exposure



[5th final information report for IEA AMF Annex XXXIII, AFHB Sept. 2010, A1]

Toxicological tests – endpoints (examples)

LDH: estimation of membrane integrity which indicates cell viability (toxic conditions → leaky membrane → cytosolic proteins (as LDH) can leave the cell); more LDH – more potential of destroying cells.

WST-1: chemical which is used to measure proliferative ability of cells (do they grow as fast as, expected?) and cell viability. WST-1 is cleaved by mitochondrial activity in viable (healthy) cells and the product (formazan) can be detected colorimetrically. Mitochondrial activity is indicative for the metabolic functioning of a cell; more of the product formazan – cells healthier.

ATP: is a key indicator for intact metabolism (the cells 'energy storage molecule'). The ability for ATP production is strongly affected by toxic conditions; more ATP – intact metabolism, cells OK.

MTT: works in a similar way as WST-1 (also product formazan).

Hoechst: is a dye (and a method) which can get into cells and is actively exported from cells. If the cell is not well, export will not work properly and the amount of the dye in a cell therefore indicates its viability; more Hoechst in the cell – worse condition.

PI exclusion: PI (propidium iodide) is only taken up by severely damaged cells. In principle a similar approach as LDH, but the other way round. Indicates membrane integrity; more PI in the cell is a sign of damage.

Glutathion, GSH: antioxidant molecule produced by the cell, which is sacrificed to oxidative molecules instead of e.g. DNA or important proteins and is used to protect proteins by binding to oxidation susceptible sites. Depletion of reduced GSH indicates high loads of oxidizing chemical species (e.g. ROS ... reactive oxygen species) and gives an estimate of the cell's antioxidant capacity.

NADPH: in principle the same as GSH, but NADPH is a reducing molecule which is used in metabolism (in part: reduce oxidized molecules that could not be protected by GSH); more NADPH means less oxidative stress.

TNF-a, IL-xy etc: cytokines, signal molecules (proteins), used for communication of cells with each other. Measurements of these proteins show the induction of inflammatory responses. ELISA is a method for quantification of such molecules, the amount indicates the strength of responses, (quantifies the crosstalk between cells, the signal exchange in relation to inflammation).

Flow cytometry (sophisticated analysis of shape and surface of the cells):
sorts and counts cells according to their state. E.g. cells in which an inflammatory response has been activated by cytokines have certain patterns/markers molecules on their surfaces, by which they can be sorted, (quantification of the outcome of the signal exchange measured by ELISA).

RT-PCR: reverse transcriptase polymerase chain reaction (analysis of intermediate molecules, which are produced by genes as reaction to the toxic influences):
measures the activity of genes, to what extent they are used by a cell. The information about gene function (e.g. used against oxidative stress) and information about gene activity indicates cellular responses to certain stimuli. Can be used for any response to any stimulus.

Comet assay (by a special method by moving the cells through a carrier substance):
measures the integrity of DNA. The extent of DNA strand breaks, which derive from oxidizing agents, radiation, errors during the process of replication (due to inhibitory chemicals, severe metabolic distortions and many more) can be estimated.

TUNEL: measures how many DNA breaks occurred by labeling the resulting free ends by means of an optical method.

H2AX: is a histone, a protein around which DNA is wrapped in the nucleus, and is involved in the repair of double strand DNA breaks (DSBs). If DSBs are present, H2AX becomes phosphorylated - 'activated' – which can be detected and used as an estimate of the occurrence of DSBs.

Abbreviations:

LDH:	Lactate dehydrogenase
WST-1:	Water soluble Tetrazolium salt 1
ATP:	Adenosin triphosphat
MTT:	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PI:	Propidium iodid
CCK-8:	Cell counting kit-8
GSH:	reduced glutathion, antioxidant molecule
ROS:	reactive oxygen species
NADPH:	Nicotinamid adenin dinucleotid phosphat
TNF-α:	Tumor necrosis factor-alpha
IL:	Interleukin
ELISA:	Enzyme linked immunosrbent assay
RT-PCR:	reverse transcriptase polymerase chain reaction
TUNEL:	Terminal dUTP nick end labeling (dUTP = deoxyuridine triphosphate)
EMSA:	Electrophoretic mobility shift assay
H2AX:	Histon 2A family, member X

Introduction in test methodologies and some biological processes

Gene expression and proteins

1) A certain signal acts on the promoter region of a target gene
2) The signal activates the promoter, protein complexes are recruited which transcribe the gene (transcription = production of RNA from a DNA template). Depending on the gene and the signal, a certain lag time between the stimulus and the activation of the gene can be observed
3) The mRNA is processed and transported out of the nucleus
4) The mature mRNA is translated to a polypeptide by the action of ribosomes (translation = production of polypeptides from an mRNA template). Depending on the protein and the state of the cell, translation may not occur immmediately

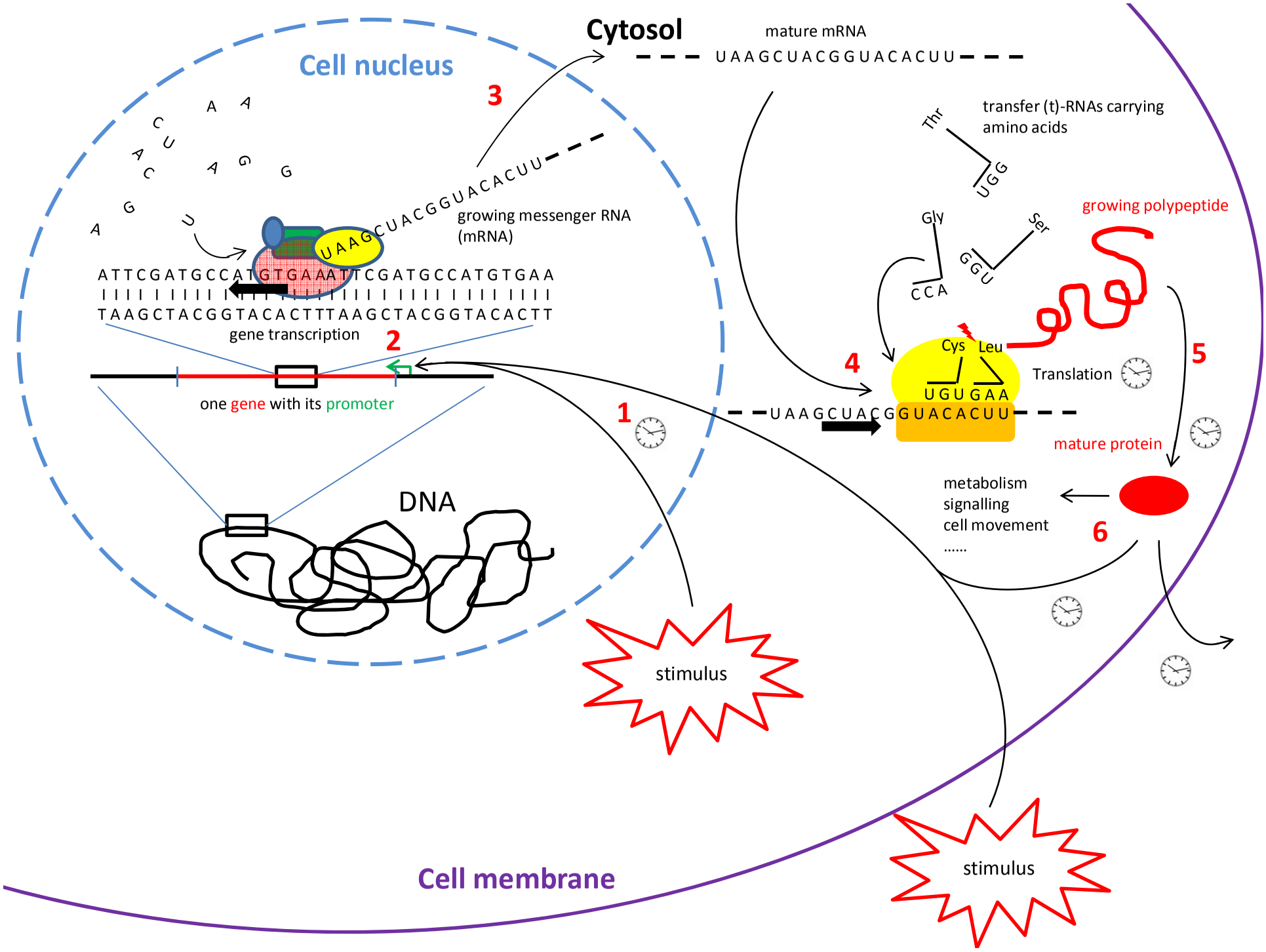
using real-time RT-PCR, we measure the amount of the mRNA of a specific gene relative to the amount of mRNA of a reference gene for which a change in expression has not to be expected upon the experimental treatment.

1) The polypeptide is processed and folded into the protein with the proper conformation. This also may take time
2) The mature polypeptide exerts its biological action, which in many cases includes the regulation of its own production. Genetic responses are often delayed (depending on the function of the protein)

Proteins can be detected:

- By quantification of the biochemical action (enzymatic activity) of a given protein in a sample, by measuring the amount of the product of the chemical reaction the protein catalyzes. LDH is detected like this

- By the use of specific antibodies which bind to the protein of interest. Chemical labels attached to the antibodies then allow quantification of how much of the protein is in a sample. This is basically how ELISA (enzyme linked immunosorbent assay) works, which we use for the quantification of TNF-a and IL-8


Cytosol
mature mRNA
UAAGCUACGGUACACUU
Cell nucleus
3
growing messenger RNA
(mRNA)
ATTCGATGCCATGTGAAATTCGATGCCATGTGAA
TAAGCTACGGTACACTTTAAGCTACGGTACACTT
gene transcription
2
one gene with its promoter
DNA
1
transfer (t)-RNAs carrying
amino acids
Thr
UGG
Gly
CCA
Ser
GGU
growing polypeptide
4
Cys
Leu
UGUGAA
Translation
5
mature protein
metabolism
signalling
cell movement
.....
6
stimulus
stimulus
Cell membrane

Cytokines (TNF-α and IL-8)

Cytokines are small soluble proteins which are released by cells in order to communicate with other cells. When cytokines bind to specific receptors (also proteins) on the cell surface, the receptor triggers a signal cascade inside the cell, finally leading to changes in the gene expression patterns and the behaviour (e.g. movement) of the cell.

Tumor necrosis factor (TNF)-α is a pro-inflammatory cytokine. It is released by cells (most importantly macrophages) upon encountering various kinds of injury and foreign material (antigens). Binding of TNF-a to TNF-receptors on a cell induces (among others) inflammatory reactions which include the production and the release of other cytokines such as IL-8

IL-8 is produced and released in response to binding of TNF-α to the TNF-receptor. It is a chemotactic factor, meaning that it attracts other cells (immune cells) to the site of injury or infection

We measure the amount of released cytokine as well as the gene expression level of the two cytokines TNF-α and IL-8. Because mRNA processing, RNA translation , protein processing and release requires time, the proteins can be detected only a certain time after gene expression has started. Furthermore, since proteins are quite stable, they can still be detected after gene expression has stopped

HMOX1 and SOD1

HMOX1 and SOD1 both are proteins involved in the defence against oxidative stress. The production of both proteins is induced by a large array of stimuli, including radiation, heat, mechanical stress, heavy metals and of course reactive oxygen species. Also nitric oxides are known to be important players. Polyaromatic hydrocarbons have been shown to act antagonizing on HMOX1 and SOD1 production.

SOD1 converts the superoxidide anion O_2^- to O_2 and H_20.

The action of HMOX1 relies in the cleavage of the biomolecule porphyrin, the products of which act anti-oxidative, anti-inflammatory and anti-apoptotic. Importantly, the production of HMOX1 is induced by inflammatory cytokines and HMOX1 induces the production of anti-inflammatory cytokines and represses the production of pro-inflammatory cytokines.

Apoptosis

Apoptosis = programmed cell death, a highly (genetically) regulated energy dependent process in which a cell undergoes a series of changes including for example the breakdown of proteins and DNA and the disintegration of the cell into multiple membrane-enclosed fragments.

This is in sharp contrast to what happens during necrosis. During necrosis, a cell is a passive victim and follows an energy independent mode of death. The cell disintegrates in an uncontrolled way, the cell membrane eventually disrupts, leading to the release of various factors into the surrounding tissue. This cell debris usually affects other cells and causes inflammation

The biological roles of apoptosis include renewal and shaping of tissues (e.g. the tissue between the fingers is eliminated during the embryonal development via apoptosis), elimination of self-intolerant immune cells, and elimination of damaged and infected cells.

FAS is a protein which is released by cells that get into contact with cell that should be eliminated. On the surface of such cells, the FAS receptor (also a protein) is present. Binding of FAS to the receptor triggers a chain of signals within the cell which finally result in apoptosis. This apoptotic pathway is referred to as the extrinsic one.

Severly damaged cells can induce their own apoptosis by signals originating from intracellular components that detect metabolic imbalances, DNA damages, and regulatory defects. This pathway is referred to as the intrinsic induction of apoptosis.

The intracellular apoptotic signal cascades of both pathways involve a large array of proteins which translate the apoptotic stimulus into the execution of apoptosis. Caspases are the most prominent group of these proteins and their production is transiently up-regulated during certain stages of apoptosis (which is also true for FAS and the FAS receptor).

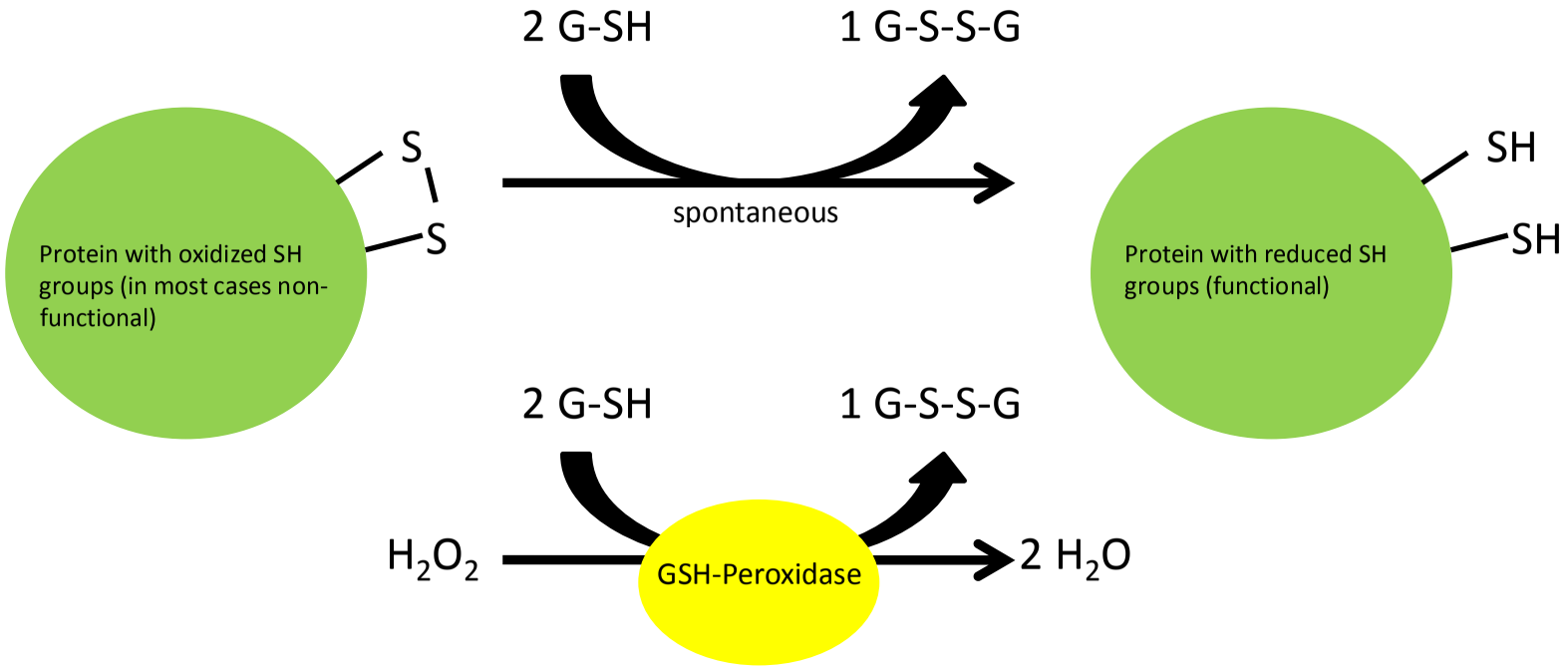
We measure apoptotic responses by real-time PCR and not on the protein level. Since the upregulation is only transient, it can happen that the peak of the expression of such pro-apoptotic genes is missed if samples are only taken at a certain time point.

Changes in the expression levels of CASPASE7 indicate an apoptotic (or anti-apoptotic) response independently on whether the intrinsic or the extrinsic pathway is active. When a change in FAS expression is detected as well, it must be assumed that the extrinsic pathway is active. No changes in FAS expression imply the activity of the intrinsic pathway.

GSH

Glutathion is a tripeptid, composed of the three amino acids glutamate, cystein and glycin.
The important feature of this molecule is the presence of a sulfhydryl (–SH) group in cystein
Its main functions are:

1) protection of SH groups in proteins from oxidation
2) detoxificatio of H_2O_2 (by the enzyme GSH-peroxidase)



The cellular pool of reduced GSH is continuously replenished by the action of the enzyme GSSG reductase. For this, NADPH (the cell's main reducing agent) is needed as an electron donor. Strongly oxidizing conditions may overburden the kinetics of GSH-peroxidase or may result in the depletion of the NADPH pool. Measurement of the concentration of reduced GSH gives a measure for how oxidative a cell experiences a certain condition.

LDH

Lactate dehydrogenase is a protein involved in the glucose metabolism and based on ist biochemical function has nothig to do with cytotoxicity. Under normal conditions, it is present in high amounts as a soluble protein in the cytosol.

Cytotoxic conditions affect the integrity of the cell membrane. This may happen directly (the cell membrane is damaged, for example by peroxidation of membrane lipids) and indirectly (the cellular membrane synthesis, maintenance and repair mechanisms are inhibitied).

LDH detection outside cell therefore gives a measure of the extent to which the membrane is damaged, which in turn is indicative for the the overall cytotoxicity of a certain treatment.

If a high LDH release is detected, it must be assumed that the cell is not able to show normal responses anymore. This is because damages in the cell membrane affect all regulatory mechanisms and the whole cellular homeostasis. Therfore, if high LDH release is observed, the cells should not be used for further endpoint measurements.

Alternative methods for the evaluation of complex aerosols Toxicity : MAETAC

Contact : Dr Jean-Paul Morin jean-paul.morin@univ-rouen.fr

This project supported by two French agencies : ADEME and ANSES aims to compare two methods employing eukaryotic biological systems (Lung slices and A549 cell lines) and a prokaryotic system (bacteria strains for Ames test) to assess the toxicity pattern of complex aerosols.

The test aerosol will be diesel combustion engine emission run on test bench under urban ARTEMIS driving cycle under variable conditions of exhaust after-treatment (oxidation catalysis and oxidation catalysis + particulate filter and fuels (reference gazole supplemented or not with rapeseed methylester).

Background :

Combustion engine emissions are a highly complex mixtures of chemicals under gaseous and particulate phases, the toxicity of which has previously been addressed by in vitro and in vivo techniques considering almost exclusively the particulate matter phase. With the progresses of emission after-treatment strategies, recent studies show an increasing importance of gas phase pollutants as major triggers of toxicity especially for oxidant stress, inflammation and gene toxicity. (Fall et al. 2007, Hasson et al. 2009, Khair et al.2009). Due to increased content of oxygenated compounds in biofuels (ethanol or seed-methylesters) increased oxidant potential of the emissions is anticipated as suggested by LePrieur et al. 2000.which may be responsible for increased mutagenic potential reported by Bünger et al. 2006-2007. MAETAC project intends to address engine emission toxicity screening using alternative methods to animal toxicity where the biological material is placed in direct contact with a continuous flow of continuously generated and conditioned diesel engine emissions to best mimic inhalation exposure in vivo.

The tools used in this program have been developed in the frame of a 5FP EC program MAAPHRI which has been coordinated by Dr JP MORIN (Morin et al. 2008, Papaioannou et al. 2006) and which have been part of the FRAME report on in vitro models of inhalation toxicity and disease.

Project :

MAETAC project objectives are :

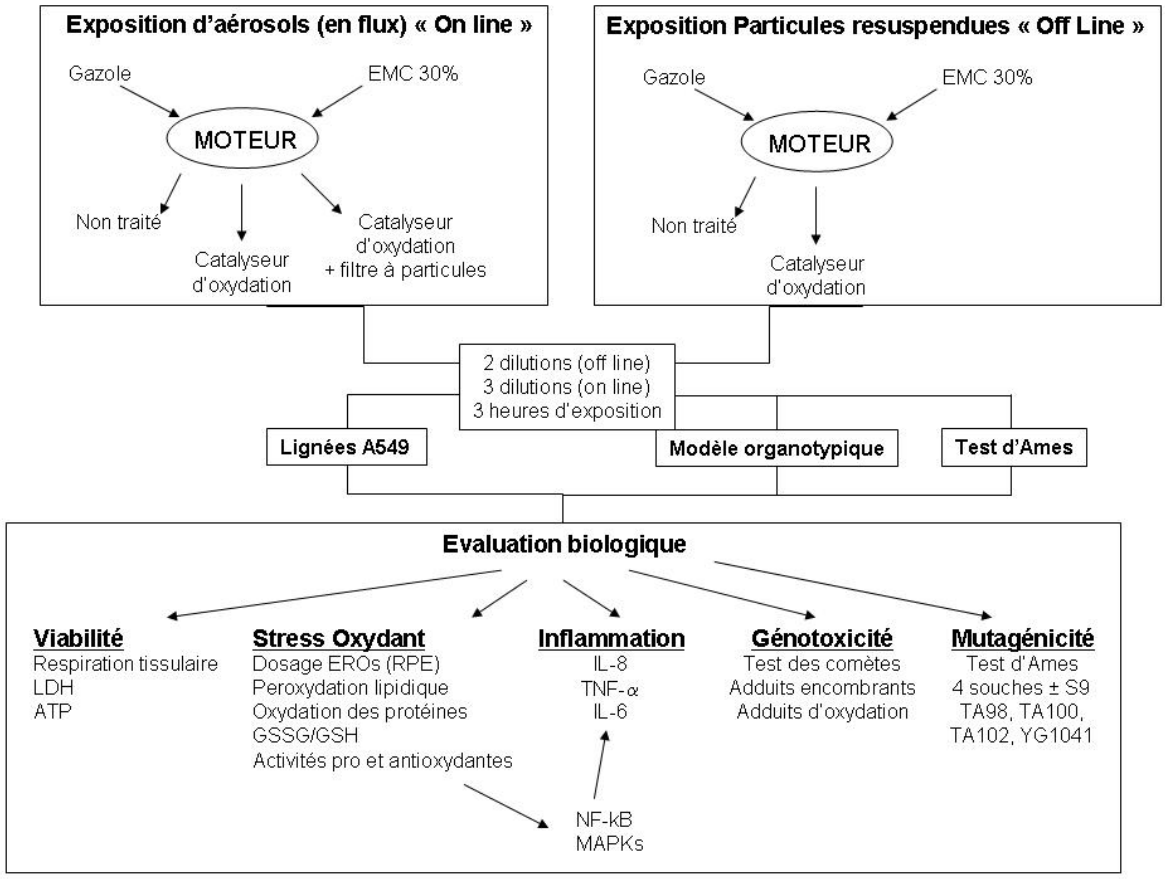
To validate pertinent in vitro methods for complex aerosol toxicity screening

To evaluate in a global aerosol approach the potential benefits of after-treatment strategies

To evaluate prospectively the potential impacts of new fuel composition especially in the field of alternative fuels like rapeseed methylesters.

These feasibility three objectives rely on the previous expertise of the consortium members namely INSERM U644, CERTAM and François Baclesse Center.

Project architecture is described in the sketch below :



Two exposures strategies will be compared : a strategy of direct exposure to continuous flows of aerosol (on line) and a strategy of exposure to the particulate matter phase resuspended after filter collection (off line). The off line procedure has been the most common approach performed since 20 years which does not allow to take gas phase pollutant into account will serve as reference.

Main toxicity pathways such as viability, oxidant stress, inflammation, gene toxicity and mutagenicity will be addressed in this project with state of the art endpoint assessment techniques.

Expected results :

Validation of in vitro screens for complex aerosol toxicity

Better understanding of after-treatment and new fuel impacts regarding health issues

Bring a complementary health based rationale to authorities for implementing new regulations for after-treatment and fuel future acceptance

Litterature :

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Papaioannou E., Konstandopoulos A., **Preterre D.**, **Morin J.P**. A selective particle sizer sampler suitable for biological exposure studies of Diesel particulate. *SAE Technical Paper* **2006**-01-175.

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A Selective Particle Size Sampler Suitable for Biological Exposure Studies of Diesel Particulate

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ABSTRACT

The objective of this study is the design, construction and evaluation of a Selective Particle Size (SPS) sampler able to provide continuous delivery of diesel soot particles of specific size ranges. The design of the sampler combines principles of aerosol transport phenomena and separation technologies. Particles smaller than a given size are removed from the exhaust by diffusional deposition, while removal of particles above a given size is achieved by low pressure inertial impaction. The main application of the developed sampler is the exposure of biological samples such as cell and tissue cultures to selected sizes of diesel exhaust particles. By applying the SPS sampler to diesel exhaust it is demonstrated that it is possible to obtain two aerosol streams with widely separated particle size distributions (of nanometric dimensions), suitable for biological exposure studies. Preliminary tests with cell cultures indicate some differences in the biological impact of smaller vs. larger diesel nanoparticles.

INTRODUCTION

Increased concern about fuel economy and emissions of greenhouse gases such as CO_2, fuels a growth in the population of diesel powered passenger cars in Europe. Advanced technology engines, emission control devices and improvement of diesel fuel are being developed to improve the environmental performance of diesel vehicles [1]. The potential benefits of these technical developments are to date evaluated almost exclusively for their capacity to reduce expected emissions on a physicochemical analysis basis. There is a lack of health effect assessment on the basis of both biological and toxicological impacts [2]. Therefore the currently prevailing approach does not guarantee that a decrease in regulated emissions will not generate compounds that might have more deleterious health effects [3].

Primary health concerns from airborne pollutants include lung carcinogenicity and non-malignant respiratory effects such as irritation, inflammation, and exacerbation or initiation of allergic hypersensitivity. The latter especially is an emerging area of concern [4]. As the prevalence of asthma and other allergic diseases has increased throughout the industrialized world in recent decades, air pollution, including exhaust emissions, especially in urban areas has been suggested as one possible cause. The Diesel engine effluent is a complex mixture of particles and gases with hundreds of chemicals, including many organics, present both in the gaseous and condensed phase.

The particle size distribution is a very pertinent factor influencing the toxic effect of exhaust emissions. The influence of particle size on the deposition rate in each region of the respiratory tract is well known. Especially particles less than 300 nm deposit with significant rates in the alveolar region. In addition it is self-evident that the composition of particles can be also distributed with respect to size. Aerosol particle size distribution can be dramatically altered by biased sampling and/or storage/resuspension of particles. This observation justifies the strategy that toxicological assays must be carried out with “real” exhaust including particulates sampled directly from the exhaust flow pipe of a running engine and maintained in suspension in the diluted gases in order to minimize changes of Particulate Matter (PM) physicochemical properties and pollutant bio-availability [5]. Measuring exposure to diesel exhaust aerosol is challenging due to the physical characteristics and chemical complexity of particulate matter: With a mean diameter of ~100 nm Diesel PM is composed primarily of organic elemental carbon, adsorbed and condensed hydrocarbon, sulfate and metals [6] The ratio of organic to inorganic carbon depends upon a number of factors that include fuel, engine type, duty cycle, engine maintenance, operator habits, use of emission control devices, and lubricant oil consumption [7]. Research is highly required to assess the actual fate and bioreactivity of exhaust components: particles and also

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Prevalidation of in vitro continuous flow exposure systems as alternatives to in vivo inhalation safety evaluation experimentations: Outcome from MAAPHRI-PCRD5 research program

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Abstract

Diesel engine emission aerosol-induced toxicity patterns were compared using both in vitro (organotypic cultures of lung tissue) and in vivo experimentations mimicking the inhalation situation with continuous aerosol flow exposure designs.

Using liquid media resuspended diesel particles, we show that toxic response pattern is influenced by the presence of tensioactive agent in the medium which alter particle-borne pollutant bioavailability.

Using continuous aerosol exposure in vitro, we show that with high sulfur fuel (300 ppm) in the absence of oxidation catalysis, particulate matter was the main toxic component triggering DNA damage and systemic inflammation, while a very limited oxidant stress was evidenced. In contrast, with ultra-low sulfur fuel in the presence of strong diesel oxidation catalysis, the specific role of particulate matter is no longer evidenced and the gas phase then becomes the major component triggering strong oxidant stress, increased NO_2 being the most probable trigger.

In vivo, plasma tumor necrosis factor alpha (TNFalpha), lung superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) activity levels varied in agreement with in vitro observations. Diesel emission treatment with oxycat provokes a marked systemic oxidant stress. Again NO_2 proved to account for a major part of these impacts. In conclusion, similar anti-oxidant responses were observed in in vitro and in vivo experiments after diesel emission aerosol continuous flow exposures. The lung slice organotypic culture model-exposed complex aerosol appears to be a very valuable alternative to in vivo inhalation toxicology experimentations in rodents.

Keywords: In vitro alternatives; Inhalation toxicology; Cell cultures; Lung; Aerosol exposure; Diesel exhausts; Oxidant stress; Inflammation

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EXHAUST TOXICOLOGICAL PROFILES FROM DIRECT INJECTION ENGINE WITH AND WITHOUT DIESEL PARTICULATE FILTER REGENERATION DURING NEDC CYCLING

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ABSTRACT

European regulations have made the use of diesel particulate filter (DPF) unavoidable because all future diesel vehicles have to comply with the Euro 5 regulation regarding particulate matter emissions. Indeed, DPF has an overall excellent filtering efficiency but should be periodically regenerated. We propose here an *in vitro* comparative toxicological study of diluted sampled exhaust, emitted during legislative NEDC (New European Driving Cycle) cycles with or without a DPF regeneration phase. Pollutants, particle sizing, ESR (Electron Spin Resonance) measurement and post-exposure biological evaluation were monitored. Only TNFα (Tumor Necrosis Factor alpha), a biological molecule produced during inflammatory processes, was slightly induced for the highest exhaust concentration including regeneration phase. In conclusion, it appears that regeneration process does not induce an acute toxicity.

INTRODUCTION

Numerous studies suggest that cardio-respiratory affections incidence could be linked to environmental pollution and especially to traffic-related emissions. Among regulated pollutants, particulate matter (PM) seems to play a major role in the observed effects [1,2,3,4]. Thus, in order to improve air quality and health impact, European regulation recommends reduction of regulated pollutants (THC, CO, NOx, PM) emitted from passenger cars and light-duty vehicles. Then, next Euro 5 regulation will impose DPF use on every new diesel vehicle to ensure PM emission conformity (5mg/km). However, DPF technology implies a periodic regeneration process to burn the accumulated soot in the trap in order to avoid engine performance losses. This regeneration phase could emit deleterious compounds and should be investigated in term of health impact.

The aim of this study is to determine whether DPF regeneration would biologically impact an *in vitro* rat lung slice organotypic model. Ensuring biological exposure conditions and regeneration occurrence reproducibility entailed methodological choices such as DPF controlled loading, manual regeneration triggering as well as engine running on well-defined legislative NEDC cycle in a cell test bench. Hence, *in vitro* biological model was submitted to a continuous flow of diluted exhaust (1%, 5% and 10%) for 3 hours with or without a single DPF regeneration event to be as relevant as possible to real world exposure conditions. Biological end-points parameters such as tissue viability, oxidative stress, pro-inflammatory cytokine release and exhaust oxidant potential were investigated. Engine parameters and pollutants emissions were constantly monitored.

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Toxicological characterization of diesel engine emissions using biodiesel and a closed soot filter

Ingeborg M. Kooter*, Marcel A.T.M. van Vugt, Aleksandra D. Jedynska, Peter C. Tromp, Marc M.G. Houtzager, Ruud P. Verbeek, Gerrit Kadijk, Mariska Mulderij, Cyrille A.M. Krul

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ABSTRACT

This study was designed to determine the toxicity (oxidative stress, cytotoxicity, genotoxicity) in extracts of combustion aerosols. A typical Euro III heavy truck engine was tested over the European Transient Cycle with three different fuels: conventional diesel EN590, biodiesel EN14214 as B100 and blends with conventional diesel (B5, B10, and B20) and pure plant oil DIN51605 (PPO). In addition application of a (wall flow) diesel particulate filter (DPF) with conventional diesel EN590 was tested. The use of B100 or PPO as a fuel or the DPF reduced particulate matter (PM) mass and numbers over 80%. Similarly, significant reduction in the emission of chemical constituents (EC 90%, (oxy)-PAH 70%) were achieved. No significant changes in nitro-PAH were observed. The use of B100 or PPO led to a NOx increase of about 30%, and no increase for DPF application. The effects of B100, PPO and the DPF on the biological test results vary strongly from positive to negative depending on the biological end point. The oxidative potential, measured via the DTT assay, of the B100 and PPO or DPF emissions is reduced by 95%. The cytotoxicity is increased for B100 by 200%. The measured mutagenicity, using the Ames assay test with TA98 and YG1024 strains of *Salmonella typhimurium* indicate a dose response for the nitroarene sensitive YG1024 strain for B100 and PPO (fold induction: 1.6). In summary B100 and PPO have good potential for the use as a second generation biofuel resulting in lower PM mass, similar to application of a DPF, but caution should be made due to potential increased toxicity. Besides regulation via mass, the biological reactivity of exhaust emissions of new (bio)fuels and application of new technologies, needs attention. The different responses of different biological tests as well as differences in results between test laboratories underline the need for harmonization of test methods and international cooperation.

1. Introduction

The world wide increased production of greenhouse gases such as CO_2 and accompanying climate change has heightened the need for renewable energy forms. Biofuel, such as pure plant oil (PPO[1]), biodiesel (FAME), hydrotreated vegetable oil (HVO), are renewable energy sources since they can be derived from recently living organisms, unlike other natural resources such as petroleum and coal. Although the use of biofuels involves a burning process that produces emissions, such as CO_2 and particulate matter (PM), these quantities are usually far less than those emitted by fossil energy forms.

Biodiesel is the most common biofuel and can be produced from oils or fats from biological sources, plant or animal, using trans-esterification. Oils (triglycerides) react with methanol producing biodiesel, for which the chemical name is fatty acid methyl ester (FAME) or fatty acid ethyl ester (FAEE), and glycerol. Also pure plant oil (PPO) form can be used as a biofuel. The use of PPO is generally not supported by the vehicle and engine manufacturers though, because it may cause damage to the engine. Biodiesel is only generally supported in low blends with standard diesel fuel: up to B7 or B10 (i.e. 7% or 10% biodiesel share). Higher blends are often allowed by heavy-duty engine manufacturers, but with some precautions such as increased fuel filter size.

Many studies about the change of (primarily regulated) engine exhaust emissions with biofuels have been done. Verbeek et al.

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[1] B100: biodiesel; DPF: diesel particulates filter; CVS: constant volume sampler; DTT: dithiothreitol; EC: elemental carbon; EGR: exhaust gas recirculation; ELPI: electrical low pressure impactor; ETC: European transient cycle; FAEE: fatty acid ethyl ester; FAME: fatty acid methyl ester; HC: hydrocarbon; HO-1: heme oxygenase-1; HVO: hydrotreated vegetable oil; LDH: lactate dehydrogenase; PAH: polycyclic aromatic hydrocarbon; PM: particulate matter; PPO: pure plant oil; RME: rapeseed oil methyl esters; RSO: rapeseed oil; VOC: volatile organic hydrocarbons.

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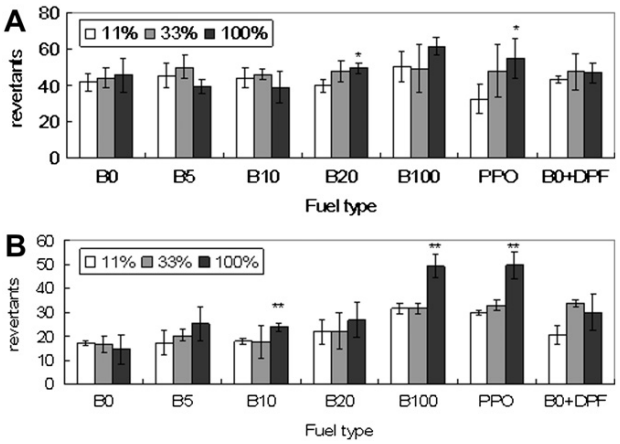

Fig. 6. Number of revertants of PM emissions for the fuel types and blends tested in absence of S9 (A: TA98 strain, B: YG1024 strain). Concentrations tested were 11%, 33% and 100%. Each bar represents 3 ETC. Error bars indicate the standard error of the mean. *, ** significantly different from the 11% sample at $P < 0.05$, <0.01 respectively.

used as part of a standard genotoxicity testing battery. Discrepancies in literature have been described about the mutagenic potency of PPO and FAME. Whereas rapeseed oil methyl esters (RME) showed lower mutagenic potency compared to diesel fuel (Bunger et al., 1998, 2000), the use of RSO as a diesel fuel results in strong increase in mutagenicity up to a factor 60 (Bunger et al., 2007). In addition another study by Bunger raised the concern about the use of oxidation catalytic converter due to the increase of direct mutagenicity (Bunger et al., 2006). In this study we performed a full comparison of different exhaust gas emissions of biodiesel (B100), various blends of biodiesel (B5, B10, and B20), PPO and the application of the diesel particulates filter by mutagenicity. As indicated in Fig. 6 in the absence of S9 a 60% increase in the number of revertants (1.5 fold induction) was observed for both B100 and PPO compared to B0 in specifically the YG1024 Salmonella strain, but not the TA98. This strain of Salmonella typhimurium YG1024 is a derivative of the commonly used TA98 and has a high level of N-hydroxyarylamine O-acetyltransferase activity, making it highly sensitive for aromatic amines and nitroarenes. An increase in NOx is observed for both B100 and PPO. NOx and PAH are known to give rise to formation of nitro-PAH. It is apparent from Fig. 3 that the observed decrease in mass when replacing B0 for B100 or PPO is larger for total PAH and oxy-PAH then for nitro-PAH.

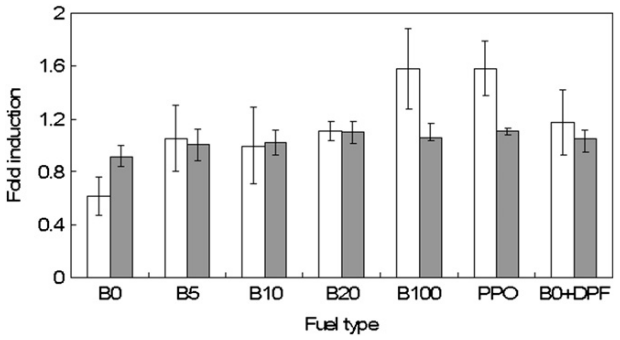

Fig. 7. Fold induction YG1024 mutagenicity of PM emissions for the fuel types and blends tested. White bars: in absence of S9, grey bars: in presence of S9. Fold induction is defined as the number of revertants by fuel type divided by the number of revertants of ethanol. Numbers of revertants of B100, PPO and B0 + DPF are corrected for the number of ETC.

We suggest that the increased mutagenicity observed for B100 and PPO might be due to formation of nitro-PAH. Chemical analyses have shown that nitro-PAHs are relatively increased. While PAHs are indirect acting mutagens, and require activation by the mammalian enzyme system before mutagenicity is expressed, nitrated PAHs are strong direct acting mutagens (Helming et al., 1992).

The increase in the number of revertants measured in the Ames test needs further investigations since fold inductions in revertant numbers over the negative control are generally not considered as biological relevant if less than 2.0-fold. Below this fold increase value, the data are considered to be unreliable with respect to determining mutagenicity. However the fact that the data in this study for both B100 and PPO, consisting of material collected from 6 individual ETC runs, show 1) an increase in NOx, 2) a relative increase in nitro-PAH (compared to total PAH and oxy-PAH) and 3) a dose response for especially the YG1024 strain, might well indicate mutagenic effects due to formation of nitro-PAH.

Bunger et al. demonstrated a strong induction of mutagenicity by diesel exhaust particles extracts and condensates from combustion of RSO and mRSO in TA98 and TA100 Salmonella strains (Bunger et al., 2007). Although no dose response relationships were shown, an increase in mutagenicity up to a factor 60 compared to the reference diesel used was reported. In addition mutagenicity is found in both the absence and in the presence of metabolic activation of S9. This clear effect could not be reproduced under the conditions performed in this study.

McDonald et al reported that the chemicals most closely associated with pulmonary toxicity were different from the chemicals that were associated with bacterial mutagenicity (e.g., nitro-PAH and oxy-PAH such as quinones) (McDonald et al., 2004). Moreover they conclude that crankcase oil-derived, particle-associated organic compounds may contribute strongly to the inflammatory effects of inhaled emissions from high-emitting vehicles. Due to the suggested increase in mutagenicity and decrease in redox activity as shown by the DTT assay, our study in addition suggests that the chemicals that are associated with bacterial mutagenicity are different from the chemicals associated with inducing oxidative stress. Moreover, the present study shows that there is a need to test emissions of new (bio)fuels and technological applications on their toxicological characteristics.

4.1. International harmonization

The literature investigation revealed that measuring procedures and results with toxicity screening vary strongly between different research institutes. Also the literature and this test program show that different biological end points within one experiment often lead to opposite results (better or worse than the reference). This underlines the need for a) international harmonization and acceptance of the test method and b) guidelines about the interpretation of results in light of the health risks. The international harmonization of health screening has been started by organizing a number of workshops by the Dutch National Institute for Public Health and the Environment RIVM. From that an engine toxicity network has been formed with the aim to launch an international (probably European) project for knowledge transfer, harmonization and validation of test methods for toxicity screening.

5. Conclusions

Based on the discrepancy in the literature our research aims were to study the toxicological potential of the different particle extracts of exhaust gas emissions of diesel, biodiesel (B100), various blends of biodiesel (B5, B10, B20), PPO and the application of

a diesel particulates filter system by cytotoxicity, oxidative stress capacity and mutagenicity. From our results it can be concluded that although B100 and PPO show much lower particulate mass emissions attention should be paid to potential increased toxicity. Besides regulation of particulates mass the biological reactivity with the use of biofuels or the application of exhaust after treatment need attention because results do not correlate with mass emissions. More research is required in order to understand and clarify the knowledge gaps and potential health risks.

The investigations show that measuring procedures and results with toxicity screening vary strongly between different research institutes. This underlines the need for international harmonization on the precise biological screening methods and a systematic research concerning the influence of different (bio)fuels and engine technologies on the toxicity of engine exhaust.

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Activities & collaborations in Switzerland

1. In vitro estrogenicity of ambient particulate matter: contribution of hydroxylated polycyclic aromatic hydrocarbons

Daniela Wenger, Andreas C. Gerecke, Norbert V. Heeb, Peter Schmid, Christoph Hueglin, Hanspeter Naegeli and Renato Zenobic 2008

2. Catalytic diesel particulate filters reduce the in vitro estrogenic activity of diesel exhaust

Daniela Wenger, Andreas C. Gerecke, Norbert V. Heeb, Hanspeter Naegeli, Renato Zenobi 2008

3. Estrogene and dioxin-like activity in diesel exhaust

Wenger D, Gerecke AC, Heeb NV, Zenobi R 2006

Collaboration between:

Laboratory for Analytical Chemistry, EMPA Dübendorf (1-3)
Department of Chemistry and Applied Biosciences, ETH Zürich (1-3)
Institute of Pharmacology and Toxicology, University of Zürich-Vetsuisse (1 and 2)

Methodes: Exhaust gas sampling and sample preparation:
Entire exhaust gas sampling (gas, particles, semivolatile components) followed by extraction with organic solvents and increasing the concentration of the extract.

Biological analysis:

celline:	T47D.Luc
exposure:	Submersed cell cultures
analysis:	Estrogen Responsive–Chemically Activated Luciferase gene expression (ER-CALUX®) assay.
	Dioxin Responsive–Chemically Activated Luciferase gene expression (DR-CALUX®) assay (works like ER-CALUX, but the activation of another group of receptors is measured).

4. Long-Term Ambient Air Pollution and Respiratory Symptoms in Adults (SAPALDIA Study)

ELIZABETH ZEMP, SERGE ELSASSER, CHRISTIAN SCHINDLER, NINO KÜNZLI, ANDRÉ P. PERRUCHOUD, GUIDO DOMENIGHETTI, TULLIO MEDICI, URSULA ACKERMANN-LIEBRICH, PHILIPP LEUENBERGER, CHRISTIAN MONN, GIANFRANCO BOLOGNINI, JEAN-PIERRE BONGARD, OTTO BRÄNDLI, WERNER KARRER, ROLAND KELLER, MARTIN H. SCHÖNI, JEAN-MARIE TSCHOPP, BEA VILLIGER, JEAN-PIERRE ZELLWEGER, and the SAPALDIA Team

Collaboration between:

Institute of Social and Preventive Medicine, University of Basel
Division of Pneumology, University of Lausanne
Federal Institute of Technology, Zürich, Switzerland
The SAPALDIA Team (SAPALDIA ... Swiss cohort study on air pollution and lung disease in adults 1991-2011)

Methodes: Epidemiological study. Air pollution was monitored with the official methods in definit time and space regions. Sample of population in these regions was interviewed about the health problems of respiratory tract.

No biological analysis.

5. Exposure to Motor Vehicle Traffic and Allergic Sensitization

Catherine Wyler, Charlotte Braun-Fahrländer, Nino Künzli, Christian Schindler, Ursula Ackermann-Liebrich, Andre P. Perruchoud, Philippe Leuenberger, Brunello Wüthrich and the Swiss Study on Air Pollution and Lung Diseases in Adults (SAPALDIA) Team 2000

Collaboration between:

The Institute of Social and Preventive Medicine, University of Basel
Department of Internal Medicine, University Hospital Basel
Department of Pneumology, University Hospital Lausanne
Allergy Unit, Department of Dermatology, University Hospital Zürich

Methodes: Also an epidemiological study (see above)

6. Health Relevance of Aerosols from Biomass Combustion in Comparison to Diesel Soot Indicated by Cytotoxicity Tests

T. Nussbaumer, N. Klippel, M. Oser

Verenum, Consultants Energy Environment, Zürich, www.verenum.ch

Methodes: Exhaust gas sampling and sample preparation:
Particle mass was collected on filters. Than this collected material was introduced in a cell culture medium and resolved (as far as possible) by means of ultrasound.

Biological analysis:

cellinie:	V79 Lungen fibroblasten (Hamster)
exposition:	Submersed cell cultures
analyse:	XTT Methode (analog MTT und WST-1)

7. Cellular Responses after Exposure of Lung Cell Cultures to Secondary Organic Aerosol Particles

ANNINA GASCHEN, DORIS LANG, MARKUS KALBERER, MELANIE SAVI, THOMAS GEISER, AMIQ GAZDHAR, CLAUS - MICHAEL LEHR, MICHAEL BUR, JOSEF DOMMEN, URS BALTENSPERGER, MARIANNE GEISER

Collaboration between:

Institute of Anatomy, University of Bern, 3012 Bern,
Laboratory of Atmospheric Chemistry, Paul Scherrer Institut (PSI)
Centre for Atmospheric Science, Department of Chemistry, University of Cambridge
Division of Pulmonary Medicine, University Hospital Bern,
Department for Biopharmaceutics and Pharmaceutical Technology, University of Saarland,

Methodes: aerosol production: organic aerosols were prepared in the PSI indoor smog chamber (a part of the POLYSOA project).

Biological analysis:

cells:	epithel cells (pig or human), primary and secodary cells, cell lines, macrophages.
exposure:	on the air-liquid (except of macrophages and cell lines, those in suspension)
analysis:	elctronmicroscopy (morphology, connections between the cells), phagocytotic activity of macrophages (phagocytose = active admission of particles by the cels); LDH; Inflammatory rections; IL-6, IL-8, TNF-a, ELISA; Alveolar EpithelialWoundRepair in Vitro – assay, in which the cell culture is damaged and the healing of the injury (its dimmension) is measured over the time.

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Effects of combustion-derived ultrafine particles and manufactured nanoparticles on heart cells *in vitro*

Maria Helfenstein[a], Michele Miragoli[b], Stephan Rohr[b], Loretta Müller[a], Peter Wick[c], Martin Mohr[d], Peter Gehr[a], Barbara Rothen-Rutishauser[a,*]

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ABSTRACT

Evidence from epidemiological studies indicates that acute exposure to airborne pollutants is associated with an increased risk of morbidity and mortality attributed to cardiovascular diseases. The present study investigated the effects of combustion-derived ultrafine particles (diesel exhaust particles) as well as engineered nanoparticles (titanium dioxide and single-walled carbon nanotubes) on impulse conduction characteristics, myofibrillar structure and the formation of reactive oxygen species in patterned growth strands of neonatal rat ventricular cardiomyocytes *in vitro*. Diesel exhaust particles as well as titanium dioxide nanoparticles showed the most pronounced effects. We observed a dose-dependent change in heart cell function, an increase in reactive oxygen species and, for titanium dioxide, we also found a less organized myofibrillar structure. The mildest effects were observed for single-walled carbon nanotubes, for which no clear dose-dependent alterations of θ and dV/dt_{max} could be determined. In addition, there was no increase in oxidative stress and no change in the myofibrillar structure. These results suggest that diesel exhaust as well as titanium dioxide particles and to a lesser extent also single-walled carbon nanotubes can directly induce cardiac cell damage and can affect the function of the cells.

1. Introduction

There is evidence from a number of epidemiological studies that ambient particulate matter (PM) causes adverse health effects associated with increased pulmonary and cardiovascular mortality (Pope et al., 1995; Peters et al., 1997; Lighty et al., 2000; Schulz et al., 2005; Rückerl et al., 2007). It has been shown that PM can cause pulmonary inflammation and blood changes, such as activation of circulating blood platelets (Nemmar et al., 2003), elevation of C-reactive protein or the von Willebrand factor (Rückerl et al., 2006; Riediker et al., 2004). Particle induced pulmonary and systemic inflammation, accelerated atherosclerosis and altered cardiac autonomic function may be part of the pathophysiological pathways, linking particulate air-pollution to cardiovascular mortality (Künzli and Tager, 2005). Increased pulmonary and cardiovascular mortality has been shown to be associated with high concentrations of airborne particles (Peters et al., 1997). Recent studies indicate a specific toxicological role of inhaled combustion-derived ultrafine particles (UFP; diameter less than 0.1 μm) (Borm and Kreyling, 2004). Acute exposure of UFP in mice induces cardiac and vascular changes by promoting a prothrombotic state and by decreasing vasomotor responsiveness (Cascio et al., 2007).

In addition to the generation of UFP from combustion processes in large amounts, there are progressively more nanoparticles (NPs), defined as manufactured particulates with at least two dimensions below 0.1 μm, released into the air, into water and soil every year from other sources, i.e. nanotechnology (Mazzola, 2003; Paull et al., 2003). Also manufactured NPs have been described to be toxic (Nel et al., 2006; Oberdörster et al., 2005). Titanium dioxide (TiO_2) particles are one of the earliest industrially produced NPs which found widespread use in substances like pigments and food additives (Maynard and Michelson, 2006) and it has been shown that exposure of ultrafine TiO_2 particles in rats leads to heart problems (Nurkiewicz et al., 2004, 2008). Other important products of particular interest are carbon nanotubes (CNT) which are used in a variety of applications from molecular electronics to energy storage

Abbreviations: APA, action potential amplitude; CM, cardiomyocytes; CV, conduction velocity; CNT, carbon nanotubes; DEP, diesel exhaust particles; θ, impulse conduction velocity; LSM, laser scanning microscopy; dV/dt_{max}, maximal upstroke velocities; NP, manufactured nanoparticles; NRVM, neonatal rat ventricular myocytes; PM, particulate matter; ROS, reactive oxygen species; SWCNT, single-walled carbon nanotubes; TiO_2, titanium dioxide; TEM, transmission electron microscopy; UFP, combustion-derived ultrafine particles.

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Oxidative stress and inflammation response after nanoparticle exposure: differences between human lung cell monocultures and an advanced three-dimensional model of the human epithelial airways

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Combustion-derived and manufactured nanoparticles (NPs) are known to provoke oxidative stress and inflammatory responses in human lung cells; therefore, they play an important role during the development of adverse health effects. As the lungs are composed of more than 40 different cell types, it is of particular interest to perform toxicological studies with co-cultures systems, rather than with monocultures of only one cell type, to gain a better understanding of complex cellular reactions upon exposure to toxic substances. Monocultures of A549 human epithelial lung cells, human monocyte-derived macrophages and monocyte-derived dendritic cells (MDDCs) as well as triple cell co-cultures consisting of all three cell types were exposed to combustion-derived NPs (diesel exhaust particles) and to manufactured NPs (titanium dioxide and single-walled carbon nanotubes). The penetration of particles into cells was analysed by transmission electron microscopy. The amount of intracellular reactive oxygen species (ROS), the total antioxidant capacity (TAC) and the production of tumour necrosis factor (TNF)-α and interleukin (IL)-8 were quantified. The results of the monocultures were summed with an adjustment for the number of each single cell type in the triple cell co-culture. All three particle types were found in all cell and culture types. The production of ROS was induced by all particle types in all cell cultures except in monocultures of MDDCs. The TAC and the (pro-) inflammatory reactions were not statistically significantly increased by particle exposure in any of the cell cultures. Interestingly, in the triple cell co-cultures, the TAC and IL-8 concentrations were lower and the TNF-α concentrations were higher than the expected values calculated from the monocultures. The interplay of different lung cell types seems to substantially modulate the oxidative stress and the inflammatory responses after NP exposure.

Keywords: human epithelial airway model; monocultures; triple cell co-cultures; nanoparticles; reactive oxygen species; inflammation

1. INTRODUCTION

Epidemiological studies have shown an association between exposure to particulate matter with a diameter less than or equal to 10 μm (PM_{10}) and adverse health effects such as cardiovascular and cardiopulmonary diseases (Samet *et al.* 2000; Brunekreef & Holgate 2002; Pope *et al.* 2004*b*; Riediker *et al.* 2004). Diesel exhaust particles (DEPs) are an important constituent of PM_{10} and are the main cause of adverse health effects (Lighty *et al.* 2000; Schwartz 2000). Additionally, *in vitro* studies have shown adverse effects of combustion-derived PM_{10} in cultures of different cell types. During

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BioToxDi

Toxicity of Diesel Exhaust on Human Lung Cells *in vitro*

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In the past years, the demand for more efficient diesel engines and the more stringent diesel emission legislation evoked the development of a large number of new fuels and lubrication oils and of new technologies for exhaust after-treatment. This trend is still on-going, and since diesel engines are very likely to be used more frequently in the near future, understanding the effects of such new developments on the toxicity of the engine emissions is crucial. In a current project, we address this topic with an *in vitro* approach in which a cellular model of the human respiratory epithelium is exposed to diesel exhaust. Our results will help to take decisions about which exhaust after-treatment systems are favourable for future use and which fuels and lubrication oils reduce the toxic potential of diesel engine emissions.

The cellular model is a well established triple cell co-culture *(1-3)*, which consists of bronchial epithelial cells combined with the two most important immune cells in the human lung, i.e. macrophages (professional phagocytotic cells) and dendritic cells (professional antigen presenting cells). The distribution of the three cell types within the model reflects the structure of the human respiratory epithelium. Compared to monocultures, this co-culture model offers a closer approach to the actual *in vivo* situation in the human lung by simulating not only the structural and functional barrier against particulate antigens, but also the lung's immunological defence system. Therefore, besides the monitoring of oxidative stress, genotoxicity, cellular morphology and viability, it also allows to study inflammatory reactions, which are known to additionally influence the responses mentioned beforehand. Furthermore, our cellular model makes exposure of the cells to diesel exhaust at the air-liquid interface possible, which simulates the *in vivo* situation more adequately than the commonly used submersed cell cultures.

The exhaust exposures are conducted using a recently established and optimized exposure system for scooter emissions *(4)*. The core element of which is a cell culture chamber system developed by the group of Prof. Dr. J.-P. Morin in Rouen, France. The cell culture chamber system was integrated into an elaborate exposure system which is located in the Laboratory for Exhaust Emission Control AFHB in Nidau, Switzerland. In this system, freshly produced diluted engine exhaust can directly be exposed to the cell cultures, thereby closely simulating the on road situation. Within the system, the most relevant physical parameters (temperature, relative humidity, volume flow and carbon dioxide concentration) are tightly controlled and kept at levels close the ones that are found in the human lung. The combination of the triple-cell co-culture, the exposure at the air-liquid interface and the sophisticated exposure system renders our experimental setup highly reproducible and realistic.

Upon exposure, the cell cultures as well as the supernatants are collected and biological responses are measured. We assess cellular and epithelial morphology, cytotoxicity, oxidative stress, inflammatory and apoptotic responses.

The exposure system has also been used to investigate toxicity of exhaust from cars with and without particle filters and different exhaust after-treatments (Publication in preparation). Currently, we are using an Opel Astra for further studies. In course of the project, we will vary the installed exhaust after-treatment system (no after-treatment, particle filter or fuel-borne catalyst), the fuel (normal fossil fuel or 20 and 100% rapeseed methyl ester) and the lubrication oil (high, low and zero sulphated ash, phosphorus and sulphur). Besides the impact of the different exhaust after treatment systems, fuels and lubrication oils, the contribution of exhaust aging and nitrogen dioxide to the exhaust toxicity will be tested.

In parallel to each exposure experiment, the engine exhaust is characterized in terms of carbon monoxide, nitrogen oxides, content of hydrocarbons and elemental carbon, particle numbers and

particle diameters. Further, the quantification of potentially genotoxic compounds from integral exhaust samples collected in parallel to the cell exposure experiments is planned. Correlation of exhaust toxicity with exhaust composition will give insight into which exhaust constituents are most important regarding exhaust toxicity.
The exposure experiment with standard fuel, standard lubrication oil and no exhaust after treatment system has already been conducted and evaluated. We found that exposure to diluted diesel exhaust does not affect viability and morphology of human lung cells *in vitro*, but strongly reduces their antioxidative capacity, independently of the dose. Further, we found that upon exposure, the cells raise antioxidative and inflammatory responses in a dose dependent manner.
Exposure with a diesel particle filter has also been conducted and the data analysis is currently in progress.

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May 2011

Antwort: Info IEA AMF; Our telephone call on ALI technology

jan.knebel@item.fraunhofer.de [jan.knebel@item.fraunhofer.de]

Sent: Tuesday, September 06, 2011 11:06 AM

To: Czerwinski Jan

Dear Jan Czerwinski,

Thanks again for the interesting telephone talk about ongoing work and future perspectives of the air-liquid interphase technology.
As mentioned, at present german public authorities focus on funding projects dealing with technologies for battery electric vehicles rather than alternative fuel vehicles. We therefore have the impression, that the research activities on possible health effects caused by combustion of alternative fuels is currently rather low.
Now coming back to your question about our today's work on the field of the air-liquid technology. We work on several projects funded by public authorities or industrial partners. We investigate several biological effects (toxic, genotoxic and immunologic) of gases and aerosols on the respiratory tract. We use therefore defined cell lines, primary cells and precision cut lung slices (PCLS) as cellular targets. Meanwhile we improved the culture and exposure technique by developing our own Fraunhofer System (P.R.I.T.-ALI), which fits to our specific requirements and contains several advantages about currently available commercial systems. For example by using a technical extension, cell exposures to test atmospheres is combined to be carried out simultaneously with cell analysis by live cell fluorescence analysis. Hence defined single as well as repeated "dosing" of sub toxic levels of the test atmospheres is possible.
Beside environmental pollution another research topic is COPD (triggered by cigarette smoke) and the impact of synthetic nanoparticles on human health.
You will find some more information on our web page (http://www.item.fraunhofer.de/en/research-areas/toxicology-environmental-hygiene/in-vitro-toxicology/index.jsp).

I hope, that these information is helpful for your proposed summary on in-vitro air-liquid technology.

Best regards,

Jan

-- --
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PERGAMON

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Exposure of human lung cells to native diesel motor exhaust—development of an optimized in vitro test strategy

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Abstract

To investigate the effects of native diesel motor exhaust on human lung cells in vitro, a new experimental concept was developed using an exposure device on the base of the cell cultivation system CULTEX (Patent No. DE19801763.PCT/EP99/00295) to handle the cells during a 1-h exposure period independent of an incubator and next to an engine test rig. The final experimental set-up allows the investigation of native (chemically and physically unmodified) diesel exhaust using short distances for the transportation of the gas to the target cells. The analysis of several atmospheric compounds as well as the particle concentration of the exhaust was performed by online monitoring in parallel. To validate the complete system we concentrated on the measurement of two distinct viability parameters after exposure to air and undiluted, diluted and filtered diesel motor exhaust generated under different engine operating conditions. Cell viability was not influenced by the exposure to clean air, whereas dose-dependent cytotoxicity was found contingent on the dosage of exhaust. Additionally, the quality of exhaust, represented by two engine operating conditions (idling, higher load), also showed well-distinguishable cytotoxicity. In summary, the experimental set-up allows research on biological effects of native engine emissions using short exposure times.

Keywords: Air pollution; Complex mixtures; Diesel exhaust; Motor emissions; Particles; Human lung cells; Air/liquid interface; Cultex system

1. Introduction

Emissions of combustion engines constitute a major source of urban air pollution. Predominantly, diesel motor exhaust is suspected to cause acute and chronic adverse effects on respiratory health due to its high amount on emitted NO_x and particles. For this reason, great efforts have been made by the development of exhaust after treatment technologies, modified engine controls for a more efficient combustion process as well as changes in the composition of fuels (e.g. rapeseed oil methyl ester, RME), which are thought to improve the situation. For analysing the biological activity of such native complex atmospheres, the methodological spectrum of alternative methods is still limited. Up to now, in vitro study concepts assessing the biological effects of complex air mixtures such as diesel motor exhaust are mostly based on two simple principles: (1) sampling the particulate phase on filters followed by the investigation of the effects of suspended and/or extracted particles (Boland et al., 1999; Murphy et al., 1999; Bonvallot et al., 2000; Takizawa et al., 2000; Bai et al., 2001; Don Porto Carero et al., 2001); (2) exposure of adherent or suspended grown cells covered totally (Teague et al., 1994; Drumm et al., 1999; van Bree et al., 2000) or sequential (Mückter et al., 1998; McManus et al., 1989; Tu et al., 1995) by medium to the gaseous phase, which is modified by humidification, CO_2 or O_2 supplementation, for example. All these experimental set-ups differ to a great extent from the realistic exposure situation. They do not take into account that particles age during preparation, form aggregates different from their composition in the atmosphere or come into contact with the cells in an unphysiological way. Additionally, reactive components of the gaseous phase may first react with medium components or the material of the incubator/culture vessel before secondly their intermediate products react with the cells. Hence, these study concepts do not facilitate investigations on effects of the combination of gaseous and particle phase.

Abbreviations: HFBE 21, human foetal bronchial epithelial cell line; FCS, foetal calf serum; PBS, phosphate buffered saline.

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Cytotoxicity and Inflammatory Potential of Soot Particles of Low-Emission Diesel Engines

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We evaluated, in vitro, the inflammatory and cytotoxic potential of soot particles from current low-emission (Euro IV) diesel engines toward human peripheral blood monocyte-derived macrophage cells. The result is surprising. At the same mass concentration, soot particles produced under low-emission conditions exhibit a much higher toxic and inflammatory potential than particles from an old diesel engine operating under black smoke conditions. This effect is assigned to the defective surface structure of Euro IV diesel soot, rendering it highly active. Our findings indicate that the reduction of soot emission in terms of mass does not automatically lead to a reduction of the toxic effects toward humans when the structure and functionality of the soot is changed, and thereby the biological accessibility and inflammatory potential of soot is increased.

1. Introduction

Since the implementation of the 1970 Clean Air Act in the United States of America, progress has been made in the reduction of exhaust gas and soot emissions of light-duty and heavy-duty vehicles (passenger cars and trucks). Particulate standards for diesel engines were introduced in 1982 and were tightened in 1991, 1994, and 1998 (*1*). The European Union followed with emission standards for heavy-duty diesel engines in 1992 (Euro I), and in stiffer form in 1998 (Euro II), 2000 (Euro III), and in October 2005 (Euro IV) (*1*). All major automobile companies have developed low-emission engines as well as filters for soot particles. Research and development strategies have focused on the reduction of soot emission yet have neglected the question of how changes in soot quality may change its effect on human health. Hence, the question is: does the low-emission engine Euro IV soot pose the same health risk per unit mass as the soot produced from old engines?

The cytotoxicity and inflammatory potential of soot nanoparticles (NPs) can be assessed by in vitro studies. Macrophages constitute the primary cellular effectors of the immune response, playing a pivotal role in the detection of all foreign bodies. These cells are ubiquitously present in the mucosal and submucosal tissues (especially in the bronchial and alveolar membrane), and human macrophage primary cultures in vitro can provide a model of potential effects upon in vivo inhalation of the soot NPs. When these cells come in contact with particles or pathogens, they become activated and secrete a variety of chemical mediators of inflammation, very aggressive against foreign molecules or particles. Currently, the toxicity of NPs is a hot research topic because the increasing production of nanomaterials is likely to significantly enhance the exposure of humans to NPs (*2–4*). However, the research in the field of nanotoxicology is still at its infancy. The parameters that determine the toxicity of NPs are not known in any detail, as one can tell from the large number of review articles published recently on the topic (*5*). The parameter most frequently used as a measure of dose is the surface area. However, lung inflammation studies involving instillation of different types of carbon NPs in mice have revealed a much more complex situation: particles prepared by different techniques exhibit significant differences in surface toxicity (*5*).

The purpose of this study was to compare the cytotoxicity and the inflammatory response, in vitro, of human monocyte-derived macrophage cells (MDMs) to a Euro IV test heavy-duty diesel engine soot and to soot from an old diesel engine and to relate the results to the microstructure of these particles, previously determined in detail by means of high-resolution transmission electron microscopy and other methods of NP characterization.

2. Experimental Section

In the following, the soot from a Euro IV test heavy-duty diesel engine will be referred to as EuroIV soot; the soot from an old diesel engine operating at black smoke conditions will be referred to as BS soot. The methods of soot production and collection have been described elsewhere (*6*). Briefly, the EuroIV soot originated from a modified MAN D0836 LF-4V six cylinder engine (6.9 L displacement, 228 kW), with two-stage controlled turbocharging, an externally controlled cooled exhaust gas recirculation, and a common rail injection system. The engine was developed to fulfill the Euro IV emission standard. The engine was set for a NO_x emission of 3.3 g/kWh and a PM emission of 50 mg/kWh (European stationary cycle, ESC). The BS soot originated from a D2876 CR engine, operated at 30% load, extra-low rail pressure, and air throttling (blackening number 5). The emission rate of the BS engine is 200–600 mg/kWh. The diesel fuel used for both engines was a standard low-sulfur type, containing 78% paraffin and 22% aromatic hydrocarbons (European Norm 590). All samples were collected directly from the exhaust gas of the engine using a special particle collector that was heated to the exhaust gas temperature at the collection position (200 °C).

Transmission electron microscopy, energy-dispersive X-ray spectroscopy, and temperature programmed oxidation studies revealed that EuroIV soot contained about 10% ash from the combusted engine lubricant oil (*7*). This kind of ash was not found in BS soot. For the in vitro studies, the EuroIV and BS soot was sterilized by heating to 180 °C, washed three times in distilled water, then suspended in PBS at a stock concentration of 1 mg/mL and sonicated for 48 h before the use.

Human peripheral blood monocytes were isolated from buffy coats of healthy donors by density gradient centrifugation using lympholyte-H (Cederlane, Hornby, Ontario, Canada). The lymphocytic/monocytic fraction was then

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the soot increases. Fortunately, the microstructural features that aggravate the health risk also lead to a more effective oxidation of soot particles to CO_2, provided suitable filtering techniques are applied (*16*). Hence, the development of filtering technology must be directed toward the removal of ultrasmall particles that, per unit mass, pose a higher risk to the biosphere than the more conventional forms of large-particle soot.

Acknowledgments

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J. Krahl, J. Bünger:

Recent Activities of the Fuels Joint Research Group (FJRG), reported to International Energy Agency Advanced Motor Fuels (IEA-AMF),

Report submitted to Jan Czerwinski

Fuels Joint Research Group (FJRG) is an interdisciplinary working group consisting of engineers, technicians, chemists, biologists, and physicians who develop and test new and advanced fuels of fossil and renewable origin. These fuels are investigated for their regulated and non-regulated emissions as well as the biological effects of these emissions. Further points of interest are the influence of these fuels on the performance and the durability of engines and exhaust aftertreatment devices.
The general aim is the development and testing of clean fuels with minimal emissions and excellent compatibility with engines and aftertreatment technologies.

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Recent Activities:
Fuels from renewable resources have gained worldwide interest due to limited fossil oil sources and the possible reduction of atmospheric greenhouse gas. One of these fuels is so called biodiesel produced from vegetable oil by transesterification into fatty acid methyl esters (FAME). This fuel is a proven substitute for petroleum derived diesel fuel (DF) and was introduced to the market about 20 years ago. Meanwhile biodiesel is seldom used as a neat fuel but mainly mixed with DF to so called blends in most countries. FJRG conducted several studies on FAME and blends thereof. Generally FAME showed lower regulated emissions compared to common DF with the exception of nitrogen oxides which are increased by 5 to 10%. Of the non-limited emissions, aldehydes were increased whereas mutagenic and carcinogenic PAH were lowered. This was accompanied by a lower mutagenicity of the exhaust in the bacterial reverse mutation assay (Ames test). However, recent studies showed a very low mutagenicity of DF exhaust as well, probably caused by elimination of sulfur in present DF qualities. The cytotoxicity was slightly increased probably due to the higher amount of aldehydes in the exhaust. A paradox effect was observed for blends, especially B20. The emissions of this fuel showed a markedly increased mutagenicity compared to neat biodiesel and neat petrol DF.
In Germany and Austria, the combustion of neat vegetable oil (VO) in diesel engines was propagated some years ago and this fuel was used for truck fleets and agricultural tractors. Besides tremendous technical problems, investigations of FJRG showed that VO can lead to a strong increase of mutagenicity in the exhaust despite minimally altered regulated emissions. Based on these results, German authorities banned VO for use under roofs and underground (Technical Rule for Hazardous Substances 554, TRGS 554).

A promising new biofuel for diesel engines is hydrotreated vegetable oil (HVO). Of recent FJRG studies, HVO yielded the lowest NO_x levels and showed the lowest PAH emissions, likewise in particle extracts and the condensates. Only very weak mutagenicity was seen with HVO (publication in preparation).
In Otto engines, FJRG tested gasoline additivated with MTBE and ETBE and confirmed a reduction of hazardous constituents in the exhaust, especially benzene which is known to cause leukemia. An additional mechanistic study was performed to elucidate the mode of action of benzene toxicity using the micronucleus test with human peripheral blood mononuclear cells since the exact process leading to leukemia is still unknown.

Conclusion:
A pronounced reduction of overall engine emissions was achieved during recent years by means of improvements of the engines, the fuels and exhaust after-treatment. Low sulfur- and aromatic and high oxygen content of the fuels seem to be decisive. Consequently recent studies show less pronounced differences between biofuels (FAME, HVO) and DF. Biofuels can lead to less toxic emissions compared to DF, however certain conditions can lead to opposite effects. With regard to a comprehensive risk assessment it is urged to develop a panel of standardized and internationally accepted methods which adequately display the various possible hazards and health effects of engine exhausts.

Some selected publications out of the FJRG:

1. **Krahl J, Munack A, Schröder O, Ruschel Y, Bünger J (2010)** Ultrafine particles from a heavy duty diesel engine running on rapeseed oil methyl ester. SAE International, Journal of Fuels and Lubricants 2, 132-146
2. **Westphal GA, Krahl J, Brüning T, Hallier E, Bünger J (2010)** Ether oxygenate additives in gasoline reduce toxicity of exhausts. Toxicology 268, 198-203
3. **Westphal GA, Lichey N, Mönnich A, Taeger D, Bünger J, Hallier E (2009)** The benzene metabolite para-benzoquinone is genotoxic in human, phorbol-12-acetate-13-myristate induced peripheral blood mononuclear cells at low concentrations. Arch Toxicol 83, 721-729
4. **Krahl J, Knothe G, Munack A, Ruschel Y, Schröder O, Hallier E, Westphal G, Bünger J (2009)** Comparison of exhaust emissions and their mutagenicity from the combustion of biodiesel, vegetable oil, gas-to-liquid and petrodiesel fuels. Fuel 88, 1064-1069
5. **Krahl J, Munack A, Ruschel Y, Schröder O, Bünger J (2008)** Exhaust gas emissions and mutagenic effects of diesel fuel, biodiesel and biodiesel blends. SAE-Technical Paper Series No. 2008-01-2508, Society of Automotive Engineers, Warrendale, PA, U.S.A., pp. 1-7
6. **Krahl J, Munack A, Ruschel Y, Schröder O, Bünger J (2007)** Comparison of emissions and mutagenicity from biodiesel, vegetable oil, GTL, and diesel fuel. SAE 2007 Transactions, Journal of Fuels and Lubricants 116, 931-938
7. **Bünger J, Krahl J, Munack A, Ruschel Y, Schröder O, Emmert B, Westphal G, Müller M, Hallier E, Brüning T (2007)** Strong mutagenic effects of diesel engine emissions using vegetable oil as fuel. Arch Toxicol 81, 599-603
8. **Krahl J, Munack A, Grope N, Ruschel Y, Schröder O, Bünger J (2007)** Biodiesel, rapeseed oil, gas-to-liquid, and a premium diesel fuel in heavy duty diesel engines: endurance, emissions and health effects. Clean Soil Air Water 35, 417-426

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10. **Kaack M, Weiskirch C, Eilts P (2009)**: Alkoholische Biokraftstoffe als Beimischungskomponente für konventionelle und alternative Brennverfahren. MTZ 70: 588 – 595.

11. **Kaack M, Weiskirch C, Eilts P** (2009): Alcoholic Biofuels as an Admixture Component for
Conventional and Alternative Diesel Combustion Processes. MTZ 70 (2009), issue 7/8.

12. **Krahl J, Munack A, Ruschel Y, Schröder O, Grope N, Schwarz S, Bünger J (2006)** Emissions from a heavy duty diesel engine: Gaseous compounds, particles and related health effects. In: VDI-Berichte Nr. 1958, VDI Verlag, Düsseldorf, 745–748

13. **Krahl J, Munack A, Ruschel Y, Schröder O, Schwarz S, Hofmann L, Bünger J (2006)** Influence of the phosphorus content in rapeseed oil methyl esters during a 1000 hours endurance test on the function of a SCR-System measured by exhaust gas emissions and health effects. SAE-Technical Paper Series No. 2006-01-3282, Society of Automotive Engineers, Warrendale, PA, USA, pp. 1-10

14. **Krahl J, Munack A, Schröder O, Bünger J (2005)** The influence of fuel design on the exhaust gas emissions and health effects. SAE-Technical Paper Series No. 2005-1-3772, Society of Automotive Engineers, Warrendale, PA, USA, pp. 1-6

15. **Krahl J, Munack A, Schröder O, Stein H, Herbst L, Kaufmann A, Bünger J (2005)** Fuel design as constructional element with the example of biogenic and fossil diesel fuels. Manuscript EE 04 008. Vol. VII. March, 2005, pp.1-11. http://cigr-ejournal.tamu.edu/

Research Project Report

Fuel and Technology Alternatives for Buses – Measurements with NExBTL and Jatropha Oil Methyl Ester in a Euro III Heavy Duty Engine

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Project Time Frame: December 15, 2009 – June 14, 2010

Funded by the Advanced Motor Fuels Implementing Agreement (AMF)
of the International Energy Agency (IEA)

Braunschweig, June 2010

5 Summary

Emissions of two new renewable fuels were compared to the well known emissions of mineral diesel fuel (DF) and rapeseed oil methyl ester (RME).
One fuel is a methyl ester originating from jatropha oil (JME). This oil can produced in arid areas and the production can be carried out in such a way that it is not in conflict with food production.
The second fuel is NExBTL, a hydrogenated vegetable oil. This fuel has a lower boiling curve than methyl esters, such that it is more similar to the boiling curve of DF. Therefore, it has an advantageous precondition to be suitable for engines with diesel particle filter, which are regenerated with post-injection.

Experiments were carried out on the test facilities of the Institute of Agricultural Technology and Biosystems Engineering of the Johann Heinrich von Thünen Institute (vTI) in Braunschweig, Germany. As test engine, a heavy-duty diesel engine Mercedes-Benz OM 906 LA with EURO III certification was used. This is, of course, not the most modern engine available, but it still represents the state of many of the engines that are in practical use in transport today.

The emissions of JME showed in comparison to RME better results with respect to nitrogen oxides and carbonyl emissions and with respect to mutagenicity. In contrast, this fuel had a higher emission of hydrocarbons (HC) and carbon monoxide (CO) and a higher emission of particles smaller than 300 nm. These emission trends are comparable to those of palm oil methyl ester and may be caused by less doublebonds in the fatty acids of the methyl ester and the smaller chain length (Munack et al., 2006). In contrast, due to the fatty acid characteristic, the cold filter plugging point (CFPP) is only 0 °C and therefore this fuel can only be used in warm climate.

NExBTL showed in comparison to DF similar or better results except for the carbonyl emissions. In particular, NExBTL exhibited a very low mutagenicity of the exhaust and had the lowest PAH emissions compared to the three other fuels. This trend of lower emissions had also been found for GTL fuel, which has comparable properties (Munack et al., 2005).

UCP exhaust toxicology

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Section of Environmental Health, Department of Public Health, The Faculty of Health Sciences, University of Copenhagen, Denmark, led by Professor, DMSc Steffen Loft and with Associate Professor, PhD, DMSc Peter Møller as the leading senior researcher, has many years experience in toxicological research on engine emissions. We have used in vitro models and in vivo models mainly focussing on standard reference diesel exhaust particles with some studies including ambient air particulates and most recently with particles emitted from small car engines complying with different European standards combusting standard diesel or biodiesel blends. In animals we have used exposure by inhalation, instillation and the oral route, which we find important. A list of our recent publications on in vitro and animal toxicology of combustion particles are given below. In addition we have multiple publications on effects of traffic emissions in exposed humans. We are currently working on much more advanced in vitro models including 3-D cocultures, air-liquid exposure and new functional endpoints in vitro as well as more transgenic animal models.

Recent publications on in vitro and animal studies of combustion particle toxicology:

Risom L, Dybdahl M, Møller P, Wallin H, Haug T, Vogel U, Klungland A, Loft S. Repeated inhalations of diesel exhaust particles and oxidatively damaged DNA in young oxoguanine DNA glycosylase (OGG1) deficient mice, Free Radical Res 41: 172-181, 2007

Hansen CS, Sheykhzade M, Møller P, Folkmann JK, Amtorp O, Jonassen T, Loft S. Diesel exhaust particles induce endothelial dysfunction in apoE$^{-/-}$ mice. Toxicol Appl Pharmacol 219: 24–32, 2007

Risom L, Møller P, Dybdahl M, Vogel U, Wallin H, Loft S,. Dietary exposure to diesel exhaust particles and oxidatively damaged DNA in young oxoguanine DNA glycosylase 1 deficient mice. Toxicol Lett 175: 16-23, 2007

Danielsen PH, Risom L. Wallin H, Autrup H, Vogel U, Loft S, Møller P. DNA damage in rats after a single oral exposure to diesel exhaust particles. Mutation Res 637: 49-55, 2008

Jacobsen NR, Møller P, Cohn CA, Loft S, Vogel U, Wallin H. Diesel exhaust particles are mutagenic in FE1-Muta™Mouse lung epithelial cells. Mutation Res 641: 54–57, 2008

Danielsen PH, Loft S, Møller P. DNA damage and cytotoxicity in type II lung epithelial (A549) cell cultures after exposure to diesel exhaust and urban street particles. Particle Fibre Toxicol 5:6, doi:10.1186/1743-8977-5-6, 2008.

Danielsen PH, Loft S, Kocbach A, Schwarze PE, Møller P. Oxidative damage to DNA and repair induced by Norwegian wood smoke particles in human A549 and THP-1 cell lines. Mutation Res 674: 116-122, 2009

Saber AT, Halappanavar S, Folkmann JF, Bornholdt J, Boisen AM, Møller P, Williams A, Yauk C, Vogel U, Loft S, Wallin H. Lack of acute phase response in the liver of mice exposed to diesel exhaust particles and carbon black by inhalation. Particle Fibre Toxicol 6: 12, 2009

Hemmingsen JG, Hougaard KS, Talsness C, Wellejus A, Loft S, Wallin H, Møller P. Prenatal exposure to diesel exhaust particles and effect on the male reproductive system in mice. Toxicology 264: 61-68, 2009

Danielsen PH, Loft S, Jacobsen NR, Jensen KA, Autrup H, Ravanat JL, Wallin H, Møller P. Oxidative stress, inflammation and DNA damage in rats after intratracheal instillation or oral exposure to ambient air and wood smoke particulate matter. Toxicol Sci 118: 574-585, 2010

Danielsen PH, Møller P, Jensen KA, Sharma AK, Wallin H, Bossi R, Autrup H, Mølhave L, Ravanat JL, Briedé J, de Kok T, Loft S. Oxidative stress, DNA damage and inflammation induced by ambient air and wood smoke particulate matter in human A549 and THP-1 cell lines. Chem Res Toxicol 24:168-184, 2011

Frikke-Schmidt H, Roursgaard M, Lykkesfeldt J, Loft S, Nøjgaard JK, Møller P. Effect of vitamin C and iron chelation on diesel exhaust particle and carbon black induced oxidative damage and cell adhesion molecule expression in human endothelial cells. Tox Lett 203:181-189, 2011

Hemmingsen JG, Møller P, Nøjgaard JK, Roursgård M, Loft S. Oxidative stress, genotoxicity, and vascular cell adhesion molecule expression in cells exposed to particulate matter from combustion of conventional diesel and methyl ester biodiesel blends. Environ Sci Tech resubmitted.

Toxicological research on engine exhaust, other combustion sources, perspectives, publications

Report from the Norwegian Institute of Public Health by Per E. Schwarze
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1) Recent research activities

At the Department of Air Pollution and Noise recent research has focussed on sources and components of particulate matter. Most of the work is carried out in vitro, but some of the work has also been performed in vivo, mainly in collaboration with other laboratories.

The in vitro work included particulate matter from biomass combustion, diesel exhaust, shipping emissions. Biomass combustion particles included particles from different types of combustion and different stages of combustion. Diesel exhaust PM included different diesels, diesel with or without biodiesel, with or without aftertreatment device, and also extracted and fractionated organic material (extensively characterised). In in vitro work we use both primary cells isolated from rat lung (macrophages and epithelial cells), primary cell lines (such as SAEC cells), and cell lines (different monocyte/macrophage, epithelial cells, and endothelial cells, alone or in combination).

Diesel exhaust particles

Animal studies we were involved in were carried out at RIVM in NL (Gerlofs-Nijland, Cassee, publication see below). Animals were exposed by inhalation of diesel exhaust, we contributed with analyses of possible effects in heart.

Human chamber studies were performed in Umeå (UiU; Sandstrøm). Volunteers were exposed to diesel exhaust under controlled conditions in a chamber. The analysis of signalling pathways involved in proinflammatory cytokine production showed a coherence of results when bronchial biopsies from volunteers were compared with effects in bronchial epithelial cells in vitro.

In vitro studies have been the main focus of our research. Two papers have looked at a range of inflammatory responses to different components of PM and signalling pathways involved. Among these components are traffic indicators such as 1-nitropyrene (diesel) and metals zinc and iron. Chemically very different components seem to induce a surprisingly similar range of responses, sharing several signalling pathways.

Other recent studies have investigated the responses to diesel PM with respect to increases in the carcinogen activating enzymes of P450 (e.g. CYP1A) in relation to production of pro-inflammatory cytokine induction. It seems that these responses were mutually exclusive, but that the 1A1 induction occurred at much lower concentrations (from 25ng/ml). The organic extract from these particles seemed to exert the pro-inflammatory effect, whereas residual organic compounds were responsible for the 1A1 induction. This has been further elucidated in collaboration with Arthur Braun and Alena Kubatova, and it seems that the oxidated/hydroxylated fraction is most active in the pro-inflammatory response.

In another paper particles from biodiesel (50% and 100%) combustion with or without aftertreatment were compared to ordinary diesel. Aftertreatment removed most of the mass but toxicity remained high. There were differences in the responses to diesel/biodiesel in relation to rural or urban driving cycle.

Biomass combustion particles

Characterised PM from biomass combustion from different stages of combustion was investigated. The responses were measured as cytokine production and release or release of the local anti-inflammatory, long penthraxin PTX3. Again the organic faction seemed to be the strongest to induce the responses.

2) Further projects and funding plans

A screening of many different combustion types and stages is started. The particles were collected by Christoffer Boman (Umeå) and are currently analysed by Ian Mudway (London). An application has been sent to the Research Council of Norway for funding of a project that will look at susceptibility in relation to exposure to diesel PM and components. Another application will investigate if cells from COPD patient will react to different types of particles differently from healthy controls with focus on specific mechanisms. We are still looking for a possibility for funding of more biodiesel/diesel/driving cycle/aftertreament projects.
(COPD ... chronic obstructive pulmonary disease)

3) Most recent publications concerning combustion particles and components

1. TACE/TGF-{alpha}/EGFR regulates CXCL8 in bronchial epithelial cells exposed to PM-components. Ovrevik J, Refsnes M, Totlandsdal AI, Holme JA, Schwarze PE, Låg M. Eur Respir J. 2011 May 3. [Epub ahead of print]
2. Diesel exhaust particles induce CYP1A1 and pro-inflammatory responses via differential pathways in human bronchial epithelial cells. Totlandsdal AI, Cassee FR, Schwarze P, Refsnes M, Låg M. Part Fibre Toxicol. 2010 Dec 16;7:41.
3. Pulmonary and cardiovascular effects of traffic-related particulate matter: 4-week exposure of rats to roadside and diesel engine exhaust particles.
Gerlofs-Nijland ME, Totlandsdal AI, Kilinç E, Boere AJ, Fokkens PH, Leseman DL, Sioutas C, Schwarze PE, Spronk HM, Hadoke PW, Miller MR, Cassee FR. Inhal Toxicol. 2010 Dec;22(14):1162-73.

The following manuscripts have been/ will be sent :

- Totlandsdal et al., Differential effects of the particle core and organic extract of diesel exhaust particles
- Totlandsdal et al., Differential pro-inflammatory responses induced by diesel exhaust particles with contrasting PAH and metal content
- Totlandsdal et al., Pro-inflammatory potential of different fractions of diesel engine exhaust and wood smoke particle extracts
- Gerlofs-Nijland et al., Impact of emission technology and fuel type on the oxidative and inflammatory potential of exhaust particles

Kocbach-Bølling et al., Wood smoke particles from different combustion phases induce similar pro-inflammatory effects in co-cultures.

Herseth et al., PTX3 inducd by wood smoke particles

IEA-AMF Annex XLII
Report from WNRI (Norway) on toxicity of exhaust emissions
Written by Otto Andersen, 14 September 2011
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Activities:
WNRI has been a partner in the EEA project "Influence of bio-components content in fuel on emission of diesel engines and engine oil deterioration" (BIODEG). WNRI has had the responsibility for the task "Toxicity aspects". WNRI used molecular dynamics simulations (MDS) on supercomputer for this task. From the knowledge that fossil fuel exhaust has significant presence of polycyclic aromatic hydrocarbons (PAHs) and biodiesel exhaust contain uncombusted fatty acid methyl ester (FAME), we investigated the formation of PAH-FAME complexes in blends of biodiesel in fossil diesel. This study was motivated by the assumption that formation of such complexes increase the availability of PAHs to intracellular damage. This is due to the FAME part acting to increase the membrane-crossing ability of PAHs. In other words, that FAMEs function as "vehicles" for PAHs enabling PAHs to enter into lung cells. This implies that blending biodiesel into fossil diesel might increase the exhaust toxicity. The MDS results indicated that it is likely that such PAH-FAME aggregates are being formed as nanoparticles with diameter less than 10 nm.

A presentation of this work was done at the 40th IEA-AMF ExCo meeting in Thessaloniki. A follow-up of the study was proposed in the form of a new IEA-AMF Annex. Funding for the Norwegian participants Norwegian Marine Technology Research Institute (MARINTEK) and Norwegian Institute of Public Health will be attempted secured through grants from The Norwegian Research Council. The other participants are the Biomedical Center at Uppsala University (Sweden), University of Applied Sciences in Biel (Switzerland) with work subcontracted to EMPA Analytical Laboratories, The Institute of Biomedical Research of Barcelona (Spain), Institute of Experimental Medicine AS, Prague (Czech Republic) and Department of Vehicles and Engines, Technical University Liberec (Czech Republic). Efforts will also be made to include Chemical Process Engineering Research Institute (Greece).

The online newspaper Forskning.no, which is devoted to Norwegian and international research, published a story written by WNRI on the toxicity studies (Andersen & Manzetti, 2010). This created an interest among the editors of the popular science publication "Teknisk Ukeblad " who published an article based on several interviews with Otto Andersen (Teknisk Ukeblad, 2011). This led the Norwegian state television broadcast channel NRK to produce a headline feature in the popular science program "Schrödinger's cat" about the toxicology findings on biodiesel blending (Toftaker, 2010).

The media attention of the toxicology impact of bioblending spurred the Norwegian authorities to take action. The Climate and Pollution Agency (Klif), which is a directorate under the Norwegian Ministry of the Environment, was given the task of assembling an overview of the current knowledge of the issue. Klif collaborated with The Norwegian Institute of Public Health on this. WNRI contributed to this overview with a draft version of the chapter in the book "Biodiesel: Blends, Properties and Applications" on Nova Publishers (Manzetti et al, 2011).

Another result of the increased interest in toxicity effects and particle formation in exhaust from the combustion of bioblended diesel was that The Norwegian Marine Technology Research Institute (MARINTEK) called for a meeting in Trondheim to discuss these issues. WNRI presented the problematics and their research basis and discussed related aspects with the other invited participants, which included representatives from The Norwegian Institute of Public Health, Statoil, Department of Neuroscience and Department of Cancer Research and Molecular Medicine at Faculty of Medicine at Norwegian University of Science and Technology (NTNU).

To obtain more knowledge WNRI organised the seminar "Fighting both toxic exhaust and climate gas emissions from bio-blended diesel– Assessment of strategies" in Sogndal Feb 25, 2011. Strategies were discussed for reducing toxic exhaust emissions, improving combustion and decreasing fuel consumption. The seminar brought together experts on exhaust emissions analysis, fuel properties, pre-treatment and additive technologies. Transport operators also presented their perspectives, and research needs and funding options were discussed. The seminar was streamed live onto the web page: http://www.vestforsk.no/en/news/fighting-toxic-exhaust-from-bio-blended-diesel-assessment-of-strategies.

Publications/presentations:
The toxicology study was presented as a poster at the conference Environmental Effects of Nanoparticles and Nanomaterials: 2010 (Nano2010) in Clemson, USA (Andersen et al, 2010).

A presentation of the MDS results was also made as a paper at the conference "Euro Oil & Fuel 2010. Biocomponents in Diesel fuels - impact on emission and ageing on engine oil" in Crakow, (Gilpin et al, 2010).

A review of toxicology effects of biodiesel and bioblends were published as a chapter in the book "Biodiesel: Blends, Properties and Applications" (Marchetti & Fang, 2011). The effects covered were cardiovascular diseases, lung cancer and increase in all-cause mortality in the human population. Particular focus was given to biodiesel blends and their associated adverse health effects. It was concluded that environmental health authorities worldwide are not updated with the serious nature of air pollution and that filtering technologies, fuel types and threshold values for particle content in the air are not up to date with current medical and pathophysiological findings.

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Engine exhaust toxicity research activities in the Czech Republic

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Historically, in the Czech Republic, the engine exhaust toxicity was assessed indirectly via two pathways: chemical analyses of the exhaust gas constituents, and clinical studies of individuals with varying degrees of exposure to engine exhaust in their daily lives.

Chemical analysis of exhaust gas constituents (aldehydes, polyaromatic hydrocarbons, etc.) has been carried at the Health Institute laboratories in Ústí nad Labem and in Teplice, at the Institute for Analytical Chemistry of the Czech Academy of Sciences in Brno, and at the Czech Chemical Technology Institute in Prague, with additional capacities existing at other university, state and industrial laboratories. These analyses were done on exhaust samples collected at engine testing laboratories at the Czech Technical University in Prague, at the Motor Vehicles Research Institute (now TUV-SUD Auto CZ) in Prague, and at the Technical University of Liberec.

In addition, ambient air quality studies, targeted at vehicular emissions, have been run by the Institute of Chemical Processes of the Czech Academy of Sciences, by the State Health Institute, by the Department of Natural Environment at the Charles University, by the Department of Environmental Chemistry at the Masaryk University in Brno, and others.

Genotoxicity studies and health effects studies were done primarily at the Department for Genetic Ecotoxicology at the Institute of Experimental Medicine of the Czech Academy of Sciences.

A direct assessment of the engine exhaust toxicity is a relatively new aspect in the Czech Republic. Starting in 2007, several proposals for joint projects were submitted, where samples of exhaust from engines operating on various emerging advanced fuels or retrofitted with diesel particulate filters would be sampled at the Internal Combustion Engine Laboratory at the Technical University of Liberec (TUL), and various types of toxicological assays would be conducted on those samples by the Institute of Experimental Medicine (IEM) of the Czech Academy of Sciences. From 2008, representatives of TUL and IEM have participated in the expert group focusing on harmonizing the methodology for assessment of risks of new engine technologies and new fuels to human health, organized by Miriam Gerlofs-Nijland from the Dutch Ministry of Environment (RIVM).

In 2010-2011, two sets preliminary joint experiments by IEM and TUL were carried on. In the first set, exhaust from a diesel engine powered by diesel fuel, neat biodiesel and neat heated non-esterified rapeseed oil was sampled on fluorocarbon coated filters and

polyurethane foam plugs. In the second set, similar tests were repeated on this and another engine, collecting units to tens milligrams of particulate matter per sample with high-volume samplers on 150 mm diameter Teflon membrane filters. From these samples, organic extracts were prepared, the concentrations of 16 priority polyaromatic hydrocarbons (PAH) were analyzed, and calf-thymus DNA samples have been exposed to these extracts to assess genotoxic potential of the complex mixture. As some compounds become toxic only after metabolic activation by enzymes, the DNA adduct levels were examined on samples with and without the addition of enzymes for metabolic activation (S9 microsomal fraction). The DNA adducts were analyzed by 32P-postlabelling method. Also, the concentration of 16 priority polyaromatic hydrocarbons (PAH) were analyzed.

In September 2011, IEM, TUL and the Ministry of Environment of the Czech Republic have started a new t EU funded project MEDETOX, Innovative Methods of Monitoring of Diesel Engine Exhaust Toxicity in Real Urban Traffic (LIFE10 ENV /CZ/00651). One of the main objectives of the MEDETOX is to demonstrate innovative methods to assess the possible health risk connected with the exposure of general population to diesel exhaust particles under real traffic conditions. Diesel emissions from many thousands of trucks passing big European cities represent serious health risks for general population. This is particularly true for the city of Prague (Czech Republic), where the traffic density is so high, that trucks spend long time by waiting in traffic-jams with engines turned-on. In contrast to laboratory conditions used in some previous and current studies, this project seeks to evaluate toxicity of engine exhaust during operating conditions typical for core urban areas, where the engine emissions are of highest concern as the aggregate dose is the highest. The secondary objectives are to identify health risks related to realistic everyday utilization of emerging fuels and fuel additives, to demonstrate the use of the standardized tests of toxicity as appropriate tool for regulatory decisions, and to build effective interdisciplinary network targeted at holistic assessment of health risk potential of engine exhaust during real-world operation of road vehicles and mobile machinery, and the monitoring of the effects of various policy decisions. This will be accomplished by a well-balanced team of experts on engines and emissions (TUL), toxicity assessment (IEM) and public policy (Ministry of Environment).

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The experimental set-up (Figure 1) at the University of Eastern Finland (UEF) in Kuopio, introduced in mid-2011, enables on-line exposure of cells to aerosols and analysis of related toxicological health impacts. The set-up consists of 1) different biomass-fired combustion appliances, 2) a diesel engine test bench constructed according to the ISO 8178 standard and equipped with a 30 kW eddy current dynamometer system which is capable of producing a 90 Nm torque and 14000 rpm speed (later in 2011, a chassis dynamometer designed for testing vehicles in low-to-medium performance class (Max 350 kW, 2000 Nm, 350 km/h) will be installed for vehicle emission studies), 3) different types of dilutors (ejector dilutors, porous tube dilutors, a dilution tunnel), 4) a transformation chamber made of 125 µm FEP Teflon and 30 m^3 of volume, 5) an air-liquid cell exposure unit (Vitrocell®), and 6) several instruments for measuring the physical, chemical and toxicological characteristics of the emission. All parts of the set-up are located in the same experimental hall, which minimizes sampling losses and artefacts between the different parts.

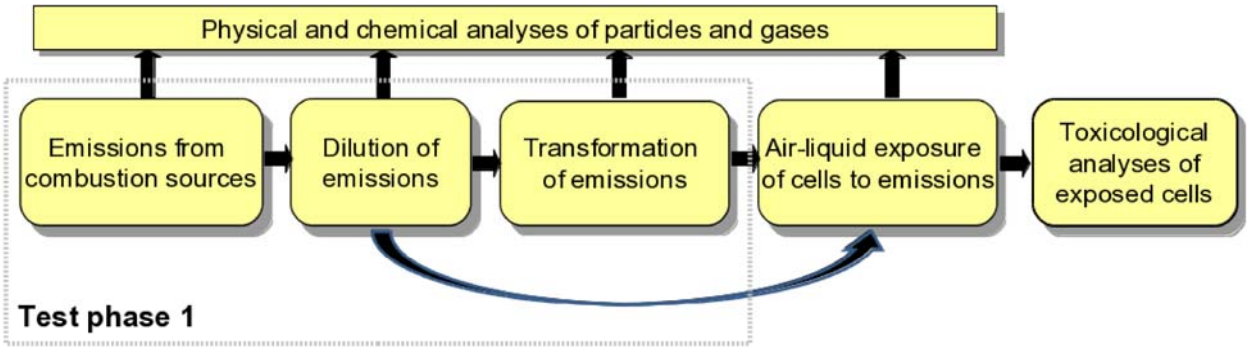

Fig. 1. The new set-up at UEF that enables on-line exposure to fresh and aged combustion aerosols

In the first test phase the deposition rate of monodisperse test aerosol particles onto the chamber walls will be determined by size distribution and number concentration measurements and model calculations. The secondary organic aerosol formation potential of diluted diesel engine emissions will be determined in the chamber both in the presence of UV light (350 nm) and in the dark, both with and without an OH (hydroxyl radical) scavenger, and with and without additional ozone and/or reactive organics. The time evolution of the physical and chemical characteristics of the diesel emission and the secondary organic aerosol yield with different initial parameters will be combined with toxicological characteristics of the same samples. Analysis of health related toxicological effects include markers of inflammation (e.g. cytokines, NO), cytotoxicity (necrosis/apoptosis), genotoxicity (e.g. DNA fragmentation) and oxidative stress in cells exposed in the air-liquid cell exposure unit.

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Engine/vehicle research competences:

- Team leader: Dr. A.G. Konstandopoulos APTL/CPERI/CERTH
- Equipment
  - engine dyno: Modern Common-rail (1.9 Lt diesel engine)
  - legal measuring procedures
  - available dilution systems
- Measuring systems (legislated, non-legislated emissions)

Gaseous emissions analyzers

- CO, CO_2, O_2, NOx, HC engine exhaust analyzers (Horiba MEXA 7400D)
- Dual NOx analyzer (Ecophysics)
- CO, CO_2 (Horiba), NOx (TEI) analyzers
- O_2 analyzer (8000M, Signal Group)
- Fast Mass Spectrometer for Exhaust Gas Analysis (Airsense 2000, V&F)
- Quadruple Mass Spectrometer (Omnistar TM, Pfeiffer)
- Multicomponent FTIR gas analyzer (Gasmet Cr-2000)
- CO, NOx, SO_2 ambient air pollution analyzers (Horiba AP-370 series)

Aerosol and particle measurement (3 nm to 1000 mm) and generation instrumentation

- Combustion Aerosol Standard (CAST, Matter Engineering)
- Phase-Doppler Analyser with Ar-ion laser (Dantec)
- Aerosizer & Aerodisperser (model 3603, TSI)
- Single Particle Counter Sizer Velocimeter (PCSV-Process Metrix)
- Scanning Mobility Particle Sizer (2 x model 3936, including Nano-DMA model 3085, TSI)
- Electric Low Pressure Impactor (ELPI, Dekati)
- Condensation Particle Counter (models 3022, 3025A, 3775, 3776, TSI)
- Photoacoustic Soot Sensor (Micro Soot Sensor model 483, AVL)
- Micro-Orifice Uniform Deposit Impactor (NanoMOUDI model 125B, MSP)
- Long Path Multiwavelength Extinction sensor (LPME, Wizard Zahoransky)
- Photoelectric Aerosol Sensor and Diffusion Charger (PAS/DC) (NanoMet system, Matter Engineering)
- Diffusion Size Classifier (DiSC, Matter Engineering)
- Mini-dilution tunnels for exhaust sampling
- Selective Particle Size (SPS) sampler

Ex-situ particle characterization facilities

- TEM with EDS
- SEM
- XRD
- C/H/N/S elemental analysis
- X-Ray micro tomography

Mobile laboratory (MOBILAB) for emissions and air pollution measurements

Benchtop micro Electron Spin Resonance Spectrometer (μESR Active Spectrum)

Toxicological analytics:

- Team leader Department of Biology, Aristotle University, Prof. Z.G. Scouras – Prof. M. Yiangou
- Exposure chambers to aerosols
    - Cultured cell lines "Air/liquid"

The Greek team is experienced in culturing mouse primary hepatocytes or alveolar macrophages after in situ perfusion of liver and lung, respectively. However, the team is not equipped to perform tissue-slices experiments.
Cell viability, inflammation and genotoxicity endpoints assayed on cultured cells at air liquid interface by Elisa – Immunochemistry – Immunofluoresence – Western analysis – Real time PCR (alternatively)

Performed projects, obtained results (part of survey)

- Multidisciplinary Approach to Airborne Pollutant Health Related Issues (MAAPHRI) (QLRT-2001-02357)
- Particulate Size and Composition Measurements for Diesel Exhaust Aftertreatment (PSICO-DEXA) (G6RD-CT-1999-00038)
- Multiwavelength Sensor for Sub-micron Particle Analysis (MULTISENS) (BRST-CT98-5537)
- Testing of Johnson Matthey small monoliths for gasoline particulate control (Service contract)
- Development of Innovative Instrumental Techniques for Coal Combustion (DITEC) (ECSC N.7220-PR/076)

Publications

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Emissions- & health activities at University of Southern California

Prof. Dr. C. Sioutas, contact: Sioutas@usc.edu

1. **Physicochemical and toxicological assessment of the semi-volatile and non-volatile fractions of PM from heavy- and light-duty vehicles operating with and without emissions control technologies**

   Funding Source: California Air Resources Board
   Role: Principal Investigator
   Project period: 01/01/2007-12/31/2011
   Specific Aims:

   1. Conduct dynamometer experiments to measure the physical, chemical and biological characteristics of PM from heavy-duty diesel vehicles with and without emission control technologies under different driving cycles.
   2. Conduct dynamometer experiments to measure the physical, chemical and biological characteristics of PM from light-duty gasoline vehicles with and without emission control technologies under different driving cycles
   3. Conduct *chemical* testing to assess redox activity and electrophilicity of fine and ultrafine PM collected with the concentrator (VACES-Biosamplers) tandem and conventional filters in Tasks 1-2.

2. **Cardiopulmonary Health Effects: Toxicity of Semi-volatile and Non volatile Components of Ultrafine PM.**

   Funding Source: California Air Resources Board (CARB)
   Role: Co-Principal Investigator (with Dr. Michael Kleinman, UC Irvine)
   Project period: 02/01/2009- 12/31/2012
   Specific Aims

   This project is aimed to determine how the toxicity of ultrafine particles depends on the concentration and characteristics of the semi-volatile and non-volatile fractions of PM emitted from vehicles and other sources. If successful this project could provide improved understanding of the mechanism of toxic action of freshly-emitted combustion aerosols and identify fractions of the aerosol causally related to health effects. This information will aid regulators and planners in developing air quality regulations and land use guidance to better protect the health of California residents.

3. **In-Vehicle Air Pollution Exposure Measurement and Modeling for Pregnant Women in the National Children's Study**

   Funding Source: California Air Resources Board (CARB)
   Role: Co-Principal Investigator (with Dr. Ralph Delfino, UC Irvine)
   Project period: 02/01/2008-12/31/2012
   Specific Aims

   The purpose of the proposed study is to collect in-vehicle air pollution data in Southern California, develop and validate in-vehicle exposure models, and apply the models to estimate in-vehicle exposure for pregnant women in the National Children's Study (NCS) cohort in Southern California. The proposed work will enhance our ability to estimate personal exposure to vehicle-related air pollutants and evaluate several main hypotheses to be tested in the Federal-Funded NCS, including:

1. Exposure to indoor and outdoor air pollution is associated with increased risk of asthma onset in children;
2. Environmental exposures interact with genes to increase the risk of asthma and wheezing in children;
3. Disparities in the prevalence, severity, and effective management of asthma by race and socioeconomic status are explained, in part, by social environmental factors and processes that influence exposure to physical environmental risk factors, psychosocial stress, and health-related behaviors;

4. **Source**

Apportionment of Carbonaceous Aerosols Using Integrated Multi-Variant and Source Tracer Techniques and a Unique Molecular Marker Data Set

Funding Source: California Air Resources Board (CARB)
Role: Co-Principal Investigator (with Dr. James Schauer, University of Wisconsin Madison)
Project period: 02/01/2008-12/31/2011
Specific Aims

This project will generate a full year of hourly organic and elemental carbon data and daily molecular markers measurements at a central site in the Los Angeles Basin. The resulting dataset will be used to apportion the contributions of primary and secondary sources on carbonaceous aerosol concentrations. A secondary objective of this study is to quantitatively determine the viability and uncertainty of using simple measurements, such as water soluble carbon, elemental carbon and water soluble potassium, as source tracers. This project will significantly reduce the uncertainty in the contributions of primary and secondary sources to carbonaceous aerosol concentrations in the Los Angeles Basin and will help identify the most cost effective strategies for source apportionment efforts associated with State SIP development and future health studies.

5. **Peripheral**

Blood Gene Expression in Subjects with Coronary Artery Disease and Exposure to Particulate Air Pollutant Components and Size Fractions

Funding Source: California Air Resources Board (CARB)
Role: Co-Principal Investigator (with Dr. Ralph Delfino, UC Irvine)
Project period: 08/01/2010-08/31/2012
Specific Aims:

To conduct a chemical speciation of organic components in indoor and outdoor accumulation mode filters (47 weeks) collected at retirement communities of 60 study subjects in CHAPS. To use the accumulation mode composition data from Task 1 and existing metals data to conduct exposure analysis and source apportionment using chemical mass balance models. To conduct an epidemiologic analysis of the relation between gene expression and exposure to particle components and source tracers from Tasks 1 and 2. Gene expression data for 42 genes selected a priori will be available from ongoing NIH, NIEHS-funded work.
This includes genes involved in oxidative stress, antioxidant defense, xenobiotic metabolism, inflammation, coagulation, and endoplasmic reticulum stress.

6. **Sources, Composition, Variability and Toxicological Characteristics of Ultrafine Particles in Southern California**

Funding Source: South Coast Air Quality Management District (SCAQMD)
Role: Principal Investigator
Project period: 01/01/2011-01/01/2014
Specific Aims:

The objective of this proposal is to provide the much needed and currently unavailable or very limited information on the relationships between UFP sources, spatial and seasonal characteristics, and toxicity in Southern California. We will identify major primary and secondary sources of UFP in 10 distinct locations of the Los Angeles basin and determine their seasonal variability. We will determine the associations of these source contributions with the redox potency of these particles.

7. **Transcriptomics, Oxidative Stress, and Inflammatory Responses to Air Pollutants**

Funding Source: NIEHS/NIH
Role: Co-Principal Investigator (PI: Dr. Ralph Delfino, UC Irvine)
Project period: 06/01/2011-06/01/2016
Specific Aims:

The objective of this proposal is to provide the much needed and currently unavailable or very limited information on the relationships between UFP sources, spatial and seasonal characteristics, and toxicity in Southern California. We will identify major primary and secondary sources of UFP in 10 distinct locations of the Los Angeles basin and determine their seasonal variability. We will determine the associations of these source contributions with the redox potency of these particles.

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Ambient Particulate Pollutants in the Ultrafine Range Promote Early Atherosclerosis and Systemic Oxidative Stress

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Abstract—Air pollution is associated with significant adverse health effects, including increased cardiovascular morbidity and mortality. Exposure to particulate matter with an aerodynamic diameter of <2.5 μm ($PM_{2.5}$) increases ischemic cardiovascular events and promotes atherosclerosis. Moreover, there is increasing evidence that the smallest pollutant particles pose the greatest danger because of their high content of organic chemicals and prooxidative potential. To test this hypothesis, we compared the proatherogenic effects of ambient particles of <0.18 μm (ultrafine particles) with particles of <2.5 μm in genetically susceptible (apolipoprotein E–deficient) mice. These animals were exposed to concentrated ultrafine particles, concentrated particles of <2.5 μm, or filtered air in a mobile animal facility close to a Los Angeles freeway. Ultrafine particle–exposed mice exhibited significantly larger early atherosclerotic lesions than mice exposed to $PM_{2.5}$ or filtered air. Exposure to ultrafine particles also resulted in an inhibition of the antiinflammatory capacity of plasma high-density lipoprotein and greater systemic oxidative stress as evidenced by a significant increase in hepatic malondialdehyde levels and upregulation of Nrf2-regulated antioxidant genes. We conclude that ultrafine particles concentrate the proatherogenic effects of ambient PM and may constitute a significant cardiovascular risk factor. **(*Circ Res*. 2008;102:0-0.)**

Key Words: air pollution ■ ultrafine particles ■ atherosclerosis ■ oxidative stress ■ HDL

It is increasingly being recognized that exposure to ambient particulate matter (PM) contributes to significant adverse health effects and is a risk factor for the development of ischemic cardiovascular events via exacerbation of atherosclerosis, coronary artery disease, and the triggering of myocardial infarctions.[1] Although this association has been documented for PM with a mean aerodynamic diameter of <10 μm (PM_{10}), there is increasing evidence that smaller particles may pose an even greater health risk. A growing literature indicates that fine particles (FPs) with an average aerodynamic diameter of <2.5 μm ($PM_{2.5}$) exert adverse health effects of greater magnitude. For example, the "Women's Health Initiative study demonstrated a 24% increase in the incidence of cardiovascular events and a 76% increase in cardiovascular mortality for every 10 $\mu g/m^3$ increase in the annual average $PM_{2.5}$ level.[2] It appears that the smallest particles that exist in the urban environment are the most dangerous.[3] Ambient ultrafine particles (UFPs) that have an aerodynamic diameter of <0.18 μm are by far the most abundant particles by number in urban environments such as Los Angeles. Because these particles are emitted mainly by vehicular emissions and other combustion sources, they contain a high content of redox-cycling organic chemicals that could be released deep into the lungs or could even spill more than into the systemic circulation. Thus, UFPs may be particularly relevant from the perspective of cardiovascular injury.[3]

In spite of the epidemiological evidence indicating that ambient PM can promote cardiovascular injury and atherosclerosis, the mechanisms of the cardiovascular injury and proatherogenic effects are not clear. However, experimental studies in susceptible animal models have shed some light on disease pathogenesis. For instance, intratracheal administration of ambient PM_{10} in Watanabe rabbits[4] or long-term exposure of apolipoprotein (apo)E-null mice to $PM_{2.5}$[5,6] enhanced atherosclerotic plaque growth. Moreover, a cross-sectional exposure study in humans showed a 5.9% increase in carotid intima–medial thickness for every 10 $\mu g/m^3$ rise in $PM_{2.5}$ levels,[7] and a prospective cohort study supported an association between long-term residential exposure to high-traffic levels of $PM_{2.5}$ and coronary atherosclerosis, as assessed by coronary artery calcification scores,[8] demonstrating that the proatherogenic effects of PM are clinically relevant.[7,8] Air pollution has also been linked to the triggering of

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that otherwise could have been larger than the 25% observed difference. Consistent with our results, it has been reported that the HDL antiinflammatory profile can be hampered by environmental factors such as the exposure to prooxidative chemicals present in cigarette smoke.[34] For example, mice exposed to second-hand smoke develop dysfunctional HDL.[35] A possible mechanism could be interference with paraoxonase and lecithin cholesterol acyltransferase activities by redox-active chemical compounds. In particular, prooxidative PM chemicals may affect critical thiol groups that are responsible for the catalytic activity of paraoxonase, leading to increased susceptibility to atherosclerosis.[36]

The fact that the FP atmosphere contains both UFPs and particles of >0.18 μm makes interpretation of those data complex. However, we have shown that the 25% difference in atherosclerotic lesion scores could be explained by the 44% increase in UFP particle number (Table and Figure 3). Total particle mass was clearly not a determining factor because the FP atmosphere had $\approx$3.9-fold greater mass than the UFP aerosol. What is likely significant is that UFPs have an $\approx$2-fold increase in the OC and PAH content on a per mass basis (Figures 1 and 2). It is possible that these prooxidative components could be delivered from a surface area that is twice as big in particles associated with the UFP atmosphere. Although we cannot claim that the PAHs are actually responsible for the lesion development, these organic chemical compounds are a good proxy for the prooxidative potential of UFPs.[13]

How do our experimental atmospheres relate to real life exposures? The particle numbers in our study were 2- to 6-fold higher than the in-vehicle exposures that commuters may encounter while traveling on Los Angeles freeways.[37] It was not logistically feasible to perform detailed dose– and time–response studies; this type of data will be important to obtain in future studies. Although it would clearly be advantageous to know the minimum exposure that is required for proatherogenic effects, previous epidemiological studies have shown that cardiovascular morbidity and mortality increase linearly without a threshold effect.[38,39] Differences in the physiology of genetically susceptible animals and humans also have to be taken into consideration when extrapolating this work to cardiovascular disease in humans.

In conclusion, we demonstrate that UFP exposures have a higher proatherogenic potential than FP exposures. These effects could be linked to a greater propensity of UFPs to generate systemic oxidative stress and to interfere with the antiinflammatory capacity of plasma HDL. Our findings are important in explaining how ambient PM may contribute to daily total and cardiovascular mortality.[40] Although such an association has been established previously for PM_{10} and $PM_{2.5}$,[2,41,42] we demonstrate that UFP exposure could be of even greater relevance. Further epidemiological and experimental data collection are required to determine the critical physicochemical and toxicological properties of UFPs in humans.

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Disclosures

None.

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Effect of Advanced Aftertreatment for PM and NO_x Reduction on Heavy-Duty Diesel Engine Ultrafine Particle Emissions

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Supporting Information

ABSTRACT: Four heavy-duty and medium-duty diesel vehicles were tested in six different aftertreament configurations using a chassis dynamometer to characterize the occurrence of nucleation (the conversion of exhaust gases to particles upon dilution). The aftertreatment included four different diesel particulate filters and two selective catalytic reduction (SCR) devices. All DPFs reduced the emissions of solid particles by several orders of magnitude, but in certain cases the occurrence of a volatile nucleation mode could increase total particle number emissions. The occurrence of a nucleation mode could be predicted based on the level of catalyst in the aftertreatment, the prevailing temperature in the aftertreatment, and the age of the aftertreatment. The particles measured during nucleation had a high fraction of sulfate, up to 62% of reconstructed mass. Additionally the catalyst reduced the toxicity measured in chemical and cellular assays suggesting a pathway for an *inverse* correlation between particle number and toxicity. The results have implications for exposure to and toxicity of diesel PM.

INTRODUCTION

In response to health concerns, regulators have promulgated ever stricter emissions standards for particulate matter (PM) from diesel engines. In the United States, the standard for PM mass from heavy duty diesel engines (HDDE) has been lowered from 1.0 g per brake horsepower-hour (g/bhp-hr) prior to 1988, to the current 0.01 g/bhp-hr for 2007 and later model year engines. These standards have been effective in lowering diesel PM mass emissions over the preceding decades. The effect on particle number emissions, chiefly ultrafine particles (UFP, defined as particles with aerodynamic diameter <100 nm), has been more nuanced, and several investigators have suggested an inverse correlation between particle mass and particle number emissions.[1,2] The research presented in this paper suggests there are a few deterministic factors which dictate whether such inverse correlation holds for a given engine/vehicle meeting the 2007 PM standard.

Prior to 2007 HDDE emissions standards were met with engine design modification and combustion process improvements. The effects of these changes on the particle emissions were to lower PM mass but possibly increase particle number emissions as suggested above. Diesel particle filters [DPF] and selective catalytic reduction [SCR] have thus far been needed to meet the 2007 PM and 2010 NO_x standards, respectively. How these devices affect particle number emissions is still the subject of some discussion, though a better understanding is emerging from this and previously published research.[3−8] DPFs very effectively filter out all solid particles, including the solid UFP emitted by uncontrolled diesels. The emissions of solid particle number and mass are therefore reduced by several orders of magnitude by DPFs. However, under certain conditions DPFs can also promote the formation of volatile sulfur-based nucleation mode particles with diameter less than 20 nm, which in certain cases can increase the total particle number emissions (i.e., when counting both solid and volatile particles). Whether or not a nucleation mode is emitted is a function of catalytic loading in the overall aftertreatment system, exhaust temperature, sulfur content of the fuel and engine oil, and previous exposure of the aftertreatment to sulfur.[3−8] The association between $PM_{2.5}$ (mass of PM with aerodynamic diameter less than 2.5 μm) and mortality and morbidity is well established,[9,10] and the reduction in diesel PM mass emissions achieved in the last two decades provides an important benefit to air quality and public health. Even so, the emissions of UFP in new low emitting HDDEs warrants further scrutiny.

The purpose of this paper is to report on the results from testing done at the California Air Resources Board's (CARB) heavy duty chassis dynamometer in Los Angeles. Four diesel vehicles, in six different aftertreatment configurations and a baseline were tested. Companion papers have reported on criteria pollutants, physical and chemical characteristics of PM, toxicity, and metals emissions measured from this testing.[3,11−14] The current analysis will focus on when and how nucleation occurs in diesel vehicles equipped with aftertreatment and correlate these events with results from measurements of PM toxicity and chemical composition. The results are significant in that they suggest catalyst in the aftertreatment, whether in the DPF or SCR, can in certain conditions contribute to both an increase in particle number *and* a decrease in measured toxicity.

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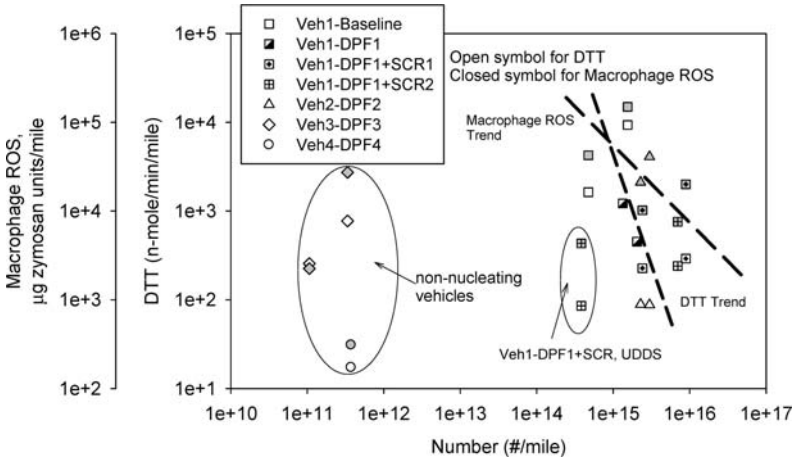

Figure 6. Total particle number and expression in the DTT acellular and Macrophage ROS in vitro assays during cruise at 50 mph and UDDS. For the nucleating configurations higher levels of catalyst in the aftertreatment leads to both higher particle number and lower expression in these two assays.

volatile, and consist of ions and OC rather than EC. They are also different from the organic based nucleation mode particles that can be emitted from uncontrolled diesel engines and that are currently observed on-road. Second, exposure to high particle number concentrations derived from diesel engines with retrofit aftertreatment for PM and NO_x control will occur mainly on or near those roads where temperatures in the aftertreatment reach the critical levels needed for nucleation, such as freeways and perhaps some major arterials or steep upgrades. These very small volatile particles have relatively short atmospheric lifetimes and are quickly removed as one moves away from the roadways.[22−24]

The smaller size and chemical composition will most likely affect the toxicity of post-2007 HDDE UFP. Figure 6 shows two measures of toxicity vs overall particle number emissions. In the current study the toxicity was determined by testing for reactive oxygen species (ROS) activity, by measuring the dithiothreitol (DTT) consumption rate[12] and by in vitro exposure to rat alveolar macrophages.[25] It has been suggested that particle number might be an indicator of toxicity. However, the particle number emissions and measures of toxicity measured in this study and shown in Figure 6 do not suggest such a relationship for either DTT or Macrophage ROS. The presence of catalytic aftertreatment, which encourages nucleation and therefore high particle number emissions, also appears to reduce the toxicity of emissions. For example the presence of catalyst effectively removes the water-soluble organics that have been shown to correlate well with DTT expression. This would explain the apparent *inverse* relationship between particle number emissions and toxicity seen in nucleating configurations in Figure 6. No sweeping conclusions can be reached from this result, and more measures of toxicity and health effects of diesel PM need to be made for a complete analysis. It does however suggest a rethinking of the health effects of particle number emissions (solid and volatile) from diesel engines.

The aftertreatment tested in the current study are mainly retrofit devices and aside from DPF3 all rely on passive regeneration, meaning that the collected soot is removed slowly and continually without introducing additional energy to the system. OEM installed DPFs are widely expected to employ both passive and active regeneration, the latter in the form of either a diesel fuel burner or diesel fuel injector installed upstream of the DPF, used to temporarily increase the temperature in short discrete events, as needed, to burn of the collected soot. During the discrete active regeneration events AT_{out} temperatures can reach >500 °C which will most likely also release sulfur stored in the DPF and lead to nucleation. These devices will likely subsequently have the capacity store sulfur and thus repress nucleation for a considerable amount of time after each active regeneration.

■ ASSOCIATED CONTENT

Supporting Information. Figure S1 shows the complete laboratory sampling setup, while Figure S2 shows the speed vs time trace of the UDDS cycle, and Figure S3 shows the particle size distribution measured in the CSV tunnel during idle. Table S1 shows the complete details of the tested vehicles, aftertreatment, and test configurations. Table S2 shows the emissions factors in mg/mi or mg/h. This material is available free of charge via the Internet at http://pubs.acs.org.

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Bio-toxicological activities at EPA related to Fuel Combustion, 30 August 2011

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Current on going studies:

1) **Controlled human exposures:** Co-exposures of human volunteers to combinations of ozone and petroleum diesel combustion emissions in a controlled chamber setting; endpoints are biomarkers of exposure (breath, blood, urine), effects (lung function, cardiac physiology, blood, exhaled breath condensate, urine), and susceptibility (specific genotypes).
2) **Controlled animal model exposures:** Examination of indicators of health effects of biodiesel and petroleum diesel combustion emissions in rodent models, with lung and cardiac physiological endpoints, blood and tissue markers; some studies with photochemistry effects on the emissions. [In collaboration with Ian Gilmour, Urmila Kodavanti, Aimen Farraj, John Offenberg, US EPA in Research Triangle Park, NC; Matt Campen, U. New Mexico, Albuquerque]
3) **In vitro controlled exposures:** In vitro screening of the biological potency of combusted biodiesel emissions (gases and particles) relative to combusted petroleum diesel emissions using human airway cells and endothelial cells.
4) **Field panel marker study:** Examination of markers of petroleum diesel combustion emission exposure and blood health effect indicators in Navy submariners.[In collaboration with Joachim Pleil, US EPA RTP, NC]

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Impacts of Advanced Fuels and Emission Control Technologies on the Toxicity of Automotive Emissions

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With automotive emission limits becoming increasingly stringent worldwide, there is an emphasis on the development and deployment of advanced fuels and technologies that enable reduction of emissions. Development of new fuel formulations and blends is also in response to a need for fuel sustainability in a global arena of increasing fuel usage and shrinking fuel resources. While these measures are important, it can also be expected that changes in fuel formulation and implementation of emission reduction technologies will alter the range of emission constituents generated with unknown human health impacts. Consequently, lower emissions may not necessarily mean cleaner emissions.

The work conducted by the Inhalation Toxicology Laboratory of Health Canada (Environmental Health Centre, Ottawa, Canada) in collaboration with the Proteomics Laboratory of Health Canada (Environmental Health Centre, Ottawa, Canada) and the Emissions Research and Measurement Section of Environment Canada (River Road Laboratories, Ottawa, Canada) aims to fill the knowledge gaps in the area of health benefits/detriments associated with the use of advanced fuels and technologies both in the light- and heavy-duty transportation sectors.

In order to assess health risks associated with use of new fuels and technologies, we are using an integrated approach consisting of assessment and mechanistic validation of toxicity employing *in vitro* and *in vivo* systems of increasing complexity and sophistication. Initial assessment of emission toxicity is assessed on extracts of vehicular emissions collected on filters using validated *in vitro* systems. Samples yielding contrasting toxicity responses (high and low) are selected for *in vivo* verification of toxicity, employing intra-pharyngeal, intranasal, intratracheal, or per os (i.e., by gavage) exposures. While our *in vitro* work provides a high-throughput, multi-factorial analytical platform to build an extensive knowledge-base of toxic potencies for a wide

array of emission materials, the *in vivo* models enable validation of these toxic potencies. Subsequent inhalation exposures to a selected subset of emission materials, is conducted in an in-house, custom-built, portable exposure facility, to allow mechanistic validation of toxicity responses and endpoints using a more realistic exposure route. Ultimately, human inhalation exposures to a narrow set of emissions will help to establish the dose-response relationships. Emphasis is also laid on the assessment of toxic potencies of and biological responses to combustion derived nanoparticles, as their contribution to overall toxicity of emission PM is poorly understood.

Our current research foci include the assessment of: 1) impacts of biodiesel blends on the toxicity of diesel emissions, 2) impacts of emission treatment (e.g. diesel oxidation catalyst, diesel particulate filter and selective catalytic reduction) on the toxicity of diesel exhaust emissions, and 3) contribution of particulate matter and gaseous components to the toxicity of whole diesel exhaust, as well as integration of these findings in toxicodynamic context to estimates of health risk.

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Regulated and Unregulated Emissions from Highway Heavy-Duty Diesel Engines Complying with U.S. Environmental Protection Agency 2007 Emissions Standards

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ABSTRACT

As part of the Advanced Collaborative Emissions Study (ACES), regulated and unregulated exhaust emissions from four different 2007 model year U.S. Environmental Protection Agency (EPA)-compliant heavy-duty highway diesel engines were measured on an engine dynamometer. The engines were equipped with exhaust high-efficiency catalyzed diesel particle filters (C-DPFs) that are actively regenerated or cleaned using the engine control module. Regulated emissions of carbon monoxide, nonmethane hydrocarbons, and particulate matter (PM) were on average 97, 89, and 86% lower than the 2007 EPA standard, respectively, and oxides of nitrogen (NO_x) were on average 9% lower. Unregulated exhaust emissions of nitrogen dioxide (NO_2) emissions were on average 1.3 and 2.8 times higher than the NO_2 emissions reported in previous work using 1998- and 2004-technology engines, respectively. However, compared with other work performed on 1994- to 2004-technology engines, average emission reductions in the range of 71–99% were observed for a very comprehensive list of unregulated engine exhaust pollutants and air toxic contaminants that included metals and other elements, elemental carbon (EC), inorganic ions, and gas- and particle-phase volatile and semi-volatile organic carbon (OC) compounds. The low PM mass emitted from the 2007 technology ACES engines was composed mainly of sulfate (53%) and OC (30%), with a small fraction of EC (13%) and metals and other elements (4%). The fraction of EC is expected to remain small, regardless of engine operation, because of the presence of the high-efficiency C-DPF in the exhaust. This is different from typical PM composition of pre-2007 engines with EC in the range of 10–90%, depending on engine operation. Most of the particles emitted from the 2007 engines were mainly volatile nuclei mode in the sub-30-nm size range. An increase in volatile nanoparticles was observed during C-DPF active regeneration, during which the observed particle number was similar to that observed in emissions of pre-2007 engines. However, on average, when combining engine operation with and without active regeneration events, particle number emissions with the 2007 engines were 90% lower than the particle number emitted from a 2004-technology engine tested in an earlier program.

IMPLICATIONS

To meet the 2007 EPA heavy-duty highway PM emissions standard, engine manufacturers have elected to equip engine exhaust with a high-efficiency C-DPF. Because of the use of the C-DPF, the PM emissions were 86% below the 2007 standard, and many unregulated gas and particle-phase emissions compounds were substantially lower than those emitted from pre-2007-technology engines. Significant air quality benefits can be expected as the C-DPF technology, or other equivalent technology, continues to be applied to future highway engines and to other nonroad and stationary diesel engines.

INTRODUCTION

Model year 2007 heavy-duty highway diesel engines sold in the United States must comply with the 2007 U.S. Environmental Protection Agency (EPA) particulate matter (PM) emission standard of 0.01 g/hp-hr, a 90% reduction from the 1994 limit of 0.1 g/hp-hr.[1] The 2007 highway engines must also comply with a phased-in oxides of nitrogen (NO_x) limit of approximately 1.2–1.5 g/hp-hr, a 38–50% reduction from the 2004 limit. This will be followed by a NO_x limit of 0.20 g/hp-hr for 2010 heavy-duty highway diesel engines. Compliance with carbon monoxide (CO) and nonmethane hydrocarbon (NMHC) emissions limits of 15.5 and 0.14 g/hp-hr, respectively, is also required.

Complying with 2007 emission limit challenges required on-highway heavy-duty diesel engines to adopt design and external equipment changes, most notably the addition of a high-efficiency catalyzed diesel particle filter (C-DPF) in the exhaust system to trap PM. A C-DPF requires periodic cleaning to prevent an unacceptable exhaust system pressure increase as the C-DPF collects PM. The cleaning process is called "regeneration" and it is achieved by several techniques. For engines in this investigation, diesel fuel injection into the diesel oxidation catalyst (DOC) or igniting a burner within the exhaust system achieved regeneration. The main goal of fuel injection or a burner is to elevate the exhaust stream temperature to oxidize soot trapped in the C-DPF to reduce engine exhaust back pressure. In addition to the exhaust

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Molecular and Isotopic Analysis of Motor Oil from a Biodiesel-Driven Vehicle

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Biodiesel, a mixture of fatty acid methyl esters (FAMEs), is increasingly recognized as a renewable fuel. While some environmental impacts of biodiesel usage have been investigated, accumulation of organic compounds in motor oil, which can subsequently leak onto roads, has not been studied. Because studies have shown that toxic polycyclic aromatic hydrocarbons (PAHs) accumulate in the motor oil of engines fueled with fossil diesel or gasoline, the objective of this study was to determine if this also occurs for engines fueled with biodiesel. Here, we sampled and analyzed motor oil of a biodiesel-powered 2005 Volkswagen Passat Wagon over 3240 km of personal-use driving. Using gas chromatography with flame ionization detection (GC−FID), we found a total of 0.5% FAMEs in the motor oil after 3240 km. We also used gas chromatography−mass spectrometry and comprehensive two-dimensional gas chromatography and did not detect PAHs or other organic compounds not present in the initial motor oil. Using natural radiocarbon analysis, a powerful technique capable of detecting biodiesel-derived carbon that would be otherwise undetectable by gas chromatography, we found a total of 0.68% biodiesel-derived carbon after 3240 km. This is similar to the amount of FAMEs found in these samples with GC−FID, indicating that the primary source of biodiesel-derived carbon in the motor oil is FAMEs (and not PAHs or other carbonaceous species). This result suggests that used motor oil of biodiesel vehicles can be less toxic based on PAH content than vehicles fueled with fossil diesel or gasoline.

Introduction

Biodiesel is a mixture of fatty acid methyl esters (FAMEs) derived from animal fats or vegetable oils. The environmental and health impacts of the biodiesel lifecycle have received considerable attention over the last 2 decades.[1−3] Large-scale implementation of biodiesel continues to be of great interest because of its potential to be a "low carbon fuel" that is nearly interchangeable with fossil diesel,[2] usually with a minimal decrease in engine power and increase in fuel consumption.[1,4−6] It also presents an opportunity to lessen the dependence upon foreign oil imports for many countries.[7,8] Some concerns exist about excess water and land use associated with producing biodiesel,[3,9−12] but efforts are underway to develop alternative sources and production techniques.[13−16] Hence, understanding the environmental impacts of biodiesel usage continues to warrant study.

To evaluate the environmental impacts of this alternative fuel in the context of factors commonly associated with the use of traditional fossil diesel, it is prudent to at least consider terrestrial and marine spills, biodegradability, exhaust emissions, and accumulation of toxic compounds in motor oil. Biodiesel biodegrades rapidly in seawater and rainwater relative to fossil diesel.[17,18] Numerous studies have focused on the regulated exhaust emissions associated with biodiesel combustion, with the general result of a reduction of most emissions but an increase in NO_x gases.[7,19,20] In one study of biodiesel use under low-speed, urban conditions and in engines that are

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An investigation on the physical, chemical and ecotoxicological characteristics of particulate matter emitted from light-duty vehicles

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PM emission reductions brought by more stringent emission standards and associated technologies may not lead to equivalent (eco-)toxicity reductions.

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ABSTRACT

Particulate matter (PM) emitted from three light-duty vehicles was studied in terms of its physico-chemical and ecotoxicological character using Microtox® bioassay tests. A diesel vehicle equipped with an oxidation catalyst emitted PM which consisted of carbon species at over 97%. PM from a diesel vehicle with a particle filter (DPF) consisted of almost equal amounts of carbon species and ions, while a gasoline vehicle emitted PM consisting of ~90% carbon and ~10% ions. Both the DPF and the gasoline vehicles produced a distinct nucleation mode at 120 km/h. The PM emitted from the DPF and the gasoline vehicles was less ecotoxic than that of conventional diesel, but not in direct proportion to the emission levels of the different vehicles. These results indicate that PM emission reductions are not equally translated into ecotoxicity reductions, implying some deficiencies on the actual environmental impact of emission control technologies and regulations.

1. Introduction

Road transportation is a major source of particulate matter (PM) in an urban environment. Particles are produced by an engine due to incomplete fuel combustion and lubricant volatilization. In addition, brake lining dust, tyre debris, and road dust contribute to vehicle-related PM emissions (Kousoulidou et al., 2008; Abu-Allaban et al., 2003). Exhaust emissions depend on a number of factors, such as the engine combustion concept and operating conditions, the fuel and lubricant, the state of maintenance, and local road conditions (Su et al., 2004). The emitted PM has been extensively investigated, in terms of emission factors (Abu-Allaban et al., 2003), size distributions (Sturm et al., 2003; Kleeman et al., 2000), source profiles (Schauer et al., 2002), molecular tracers (Fraser et al., 1999) and characterisation depending on the vehicle type (Ntziachristos et al., 2004; Thompson et al., 2004).

The control of PM from current vehicle technologies is performed either with engine measures or, more effectively, with the use of aftertreatment devices, such as catalytic converters and diesel particle filters. The use of such exhaust aftertreatment devices has led to a significant reduction in the mass of both gaseous and particulate pollutants (Twigg, 2007). However, the total particle number emissions have not been proportionally reduced. Several studies have even reported an increase in particle numbers with advanced aftertreatment systems due to enhanced nucleation downstream of such devices (Vouitsis et al., 2007; Vaaraslahti et al., 2004). Furthermore, Su et al. (2004) reported that modern engines seem to emit smaller primary particles than older ones, which may have further health implications.

The chemical footprint of exhaust emissions is also of interest when the toxic character of PM is investigated. Elemental carbon and organic matter account for most of the exhaust PM, with alkanes, polycyclic aromatic hydrocarbons (oxy-, and nitro-PAHs), fatty acids, and dicarboxylic acids being the dominant organic compounds identified (Fraser et al., 1999; Geller et al., 2006; Lim et al., 2005; Schauer et al., 2002; Valavanidis et al., 2006). Other species with potential toxic character include trace metals, and sulphate and nitrate anions (Okada et al., 2003; Kweon et al., 2002).

The complex physical and chemical character of vehicular PM calls for focused *in vivo* and *in vitro* tests and bioassays to examine

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Aktueller Wissensstand zu Gesundheitsrisiken durch Dieselmotoremissionen

Current knowledge on health hazards caused by diesel engine emissions

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1 Einleitung

Gesundheitsrisiken von Dieselmotoremissionen (DME) werden nach den bisher vorliegenden wissenschaftlichen Daten weit überwiegend durch partikuläre Abgasbestandteile und die daran anhaftenden Substanzen, vor allem polyzyklische aromatische Kohlenwasserstoffe (PAK) bestimmt. DME wurden Ende der 1980er Jahre national und international als wahrscheinlich humankanzerogen eingestuft. Grundlage waren epidemiologische Studien an beruflich in den 1950er bis 1970er Jahren Exponierten, die wegen fehlender Quantifizierung der Exposition kritisiert wurden, sowie tierexperimentelle Studien, die bei Ratten aber nicht bei anderen Nagern eine erhöhte Tumorrate ergaben.

Seit ca. 15 Jahren werden akute und chronische toxische Wirkungen von DME auf die Atemwege und das Herz-Kreislaufsystem diskutiert, wobei aktuell besonders ultrafeine Partikel, wie sie u. a. auch von Dieselmotoren emittiert werden, im Fokus stehen. Insbesondere modernen Dieselmotoren mit direkter Hochdruckeinspritzung wird eine verstärkte Emission von ultrafeinen Partikeln nachgesagt, ohne dass bislang aussagekräftige Studienergebnisse vorliegen.

In neueren Studien werden DME mit der Auslösung und Verstärkung von allergischen Symptomen in Zusammenhang gebracht. Auch hier stehen besonders die Partikel im Fokus des Interesses. Daneben wird das toxische Potenzial der Stickoxide (NO_X), die von Dieselmotoren in höheren Konzentrationen emittiert werden, als zusätzliches Risiko diskutiert.

In einem Gutachten für das Umweltbundesamt wurde 2003 behauptet, dass 10.000 bis 19.000 der jährlich 800.000 Todesfälle in Deutschland auf die Exposition durch DME zurückzuführen seien. Diese Aussage wurde stark kritisiert, da sie auf veralteten Expositionsdaten basiert.

Im Folgenden wird der Wissensstand zu kanzerogenen und nicht kanzerogenen Effekten von DME vor dem Hintergrund der technischen Entwicklung von Motoren, Kraftstoffen und Abgasnachbehandlung sowie daraus resultierender neuer Studien kritisch bewertet. Die Limitationen des aktuellen Wissensstandes und damit der Risikobewertung sowie der resultierende Forschungsbedarf werden dargestellt.

2 Exposition durch Dieselmotoremissionen

Seit Anfang der 1950er Jahre wurden in vielen Industriezweigen und im Transportwesen zunehmend stationäre und mobile Dieselmotoren verwendet. Bereiche mit besonders hohen beruflichen Expositionen von Dieselmotoremissionen (DME) finden sich im untertägigen Bergbau, im Baugewerbe, im Brücken- und Tunnelbau, bei der Eisenbahn, sowie im Transportgewerbe. Betroffen sind insbesondere Berufskraftfahrer, Maschinenführer, Bahnarbeiter, Gabelstaplerfahrer (vor allem bei Fahrten unter Dach) sowie Kfz-Mechaniker und sonstiges Instandsetzungspersonal [1]. Messungen der alveolengängigen Partikelmasse von DME an diesen Arbeitsplätzen ergaben im Median Konzentrationen von 4 bis 200 µg/m^3 Luft. Maximalwerte in besonders hoch belasteten Bereichen betrugen bis zu 2100 µg/m^3 wie z.B. in Bergwerken, in denen dieselmotorbetriebene Fahrzeuge und/oder stationäre Dieselmotoren betrieben wurden [2].

Messungen nach TRGS 554 (gemessen als elementarer Kohlenstoff) an deutschen Arbeitsplätzen ergaben in den Jahren 1985 - 1995 DME-Konzentrationen von 5 bis

1 Introduction

Health risks which arise from diesel engine emissions (DEE) are mainly caused by particulate matter and adherent constituents, especially polycyclic aromatic hydrocarbons (PAH). At the end of the 1980ies DEE were classified as human carcinogen by various national and international authorities. This classification was based on epidemiological studies concerning occupational exposures to DEE in the 1950ies until the 1970ies. These studies were criticized due to lacking exposure measurements. In addition animal experiments showed tumor induction in rats but not in other rodents. Recently as well acute and chronic toxic effects concerning the airways and the cardiovascular system are discussed. Effects which arise from ultrafine particles which can be emitted from diesel engine are especially concerned. Ultrafine particles are believed to be particularly released by modern diesel engines which are equipped with high pressure diesel direct injection. However, up to now no meaningful studies were published concerning ultrafine particles from modern diesel engines.

In recent studies DEE were associated with triggering and exacerbation of allergic symptoms. Particles are of specific interest in this context as well. In addition, DEE are discussed according to having adjuvant effects in airway sensitization. Due to cytotoxic effects nitric oxides (NO_X) from diesel engines are discussed as an additional health hazard.

A scientific expertise of the German Federal Environmental Agency dated 2003 estimated 10.000 to 19.000 additional deaths per year arising from DEE exposures. However, these estimates were criticized due to an outdated exposure data basis.

In the following the knowledge on carcinogenic and non malignant acute and chronic toxicity of DEE is discussed against the background of the development of modern diesel engines, fuels and exhaust aftertreatment. Current studies are critically assessed additionally. Limitations of the state of knowledge and resulting risk estimations are discussed as well as the urgent needs of further research.

2 Exposure towards diesel engine emissions

Since the early 1950ies many industries and transport companies made increasing use of stationary and mobile diesel engines. Frequent and high occupational exposures towards DEE occur especially in mining, in the building and construction industry as well as in the railway and transport sector. Concerned are professional drivers, machine operators, railroad workers, fork-lift operators (in particular indoors) as well as motor mechanics and maintenance workers [1]. Measurements of the respirable (fine and ultrafine) DEE particles at these workplaces showed median concentrations of 4 - 200 µg/m^3. In heavily exposed environments up to 2100 µg/m^3 were found, such as in mines in which diesel vehicles or stationary diesel engines were used [2].

Measurements according to the German TRGS 554 (technical guidelines for the handling of hazardous materials: diesel engine emissions) at German workplaces which were performed from 1985 – 1995 yielded DEE-concentrations of 5 - 130 µg/m^3 (particulate matter measured as elemen-

der Mutationen beobachtet [67]. Bei der Verwendung von Rapsöl als Kraftstoff wurden sogar sehr starke Anstiege der Mutagenität der Emissionen gemessen [70]. Eine Umrüstung im Sinne einer Zwei-Tank-Lösung und eine Ultraschallbehandlung des Kraftstoffs (E-Oil-System) ergaben keine signifikante Absenkung des mutagenen Niveaus.

5 Zusammenfassung

In den letzten 20 – 30 Jahren wurde eine überzeugende Absenkung der Dieselmotoremissionen erreicht, die das damit verbundene Gesundheitsrisiko sicherlich bedeutend abgesenkt hat. Ob auf dem derzeit erreichten niedrigen Niveau noch ein relevantes Risiko durch DME am Arbeitsplatz oder für die Allgemeinbevölkerung existiert, ist nach der vorliegenden Datenlage nicht sicher abschätzbar. Da aber entsprechende epidemiologische Studien auf der Grundlage des derzeitigen Emissionsniveaus wegen der o.g. Latenz noch nicht vorliegen können, müssen im Sinne der Prävention neue einzuführende Technologien und Kraftstoffe durch Emissionsmessungen und Kurzzeit-Screening-Tests hinsichtlich der zu erwartenden Emissions- und Risikominderung untersucht werden.

emissions was seen at rated power. This effect is probably caused by the elevated formation NO_X leading to an enhancement of nitro-PAH formation [66]. A strong short term increase of mutagenic emissions showed up during regeneration of an DPF-prototype [67]. A very strong elevation of mutations occurred using crude rapeseed oil as diesel engine fuel [70]. Using the so called two tank solution and also sonication of the fuel (E-Oil-system) did not result in a significant decrease of mutagenic effects.

5 Summary

During the last 20 – 30 years a convincing decrease of DEE was achieved which had certainly significantly reduced DEE associated health risk. However, the available data do not allow an unequivocal assessment whether the current low DEE levels still cause significant environmental or occupational health risks. Since however, the discussed improvements concerning DEE will only be epidemiologically apparent after a latency of several years, exposure measurements and short term screening test are valuable tools to monitor technical measures which are intended to minimize possible health risk of traffic emissions.

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REVIEW ARTICLE

Health effects of concentrated ambient air particulate matter (CAPs) and its components

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Abstract

We review literature that provides insights on health-related effects observed in laboratory-based inhalation studies in humans and laboratory animals using concentrated ambient air particulate matter (CAPs) in the fine, thoracic coarse, and ultrafine size ranges. The CAPs studies are highly informative on the health effects of ambient air particulate matter (PM) because they represent realistic PM exposure mixtures. When PM components are also analyzed and regressed against the effects, they can sometimes be used to identify influential individual components or source-related mixtures responsible for the effects. Such CAPs inhalation studies are analogous to epidemiological studies of human populations for which both health-related effects were observed and PM composition data were available for multi-pollutant regression analyses or source apportionment. Various acute and chronic health-related effects have occurred in short- and long-term CAPs inhalation studies in the cardiovascular, nervous, hepatic, and pulmonary systems, as well as changes in markers of the metabolic syndrome, and many correspond to effects associated with ambient air PM exposures in epidemiological studies. In addition, many CAPs studies have been conducted in coordination with *in vitro* studies that have identified biomarkers indicative of the underlying biological mechanisms that account for the responses.

Keywords: *Accumulation mode PM; CAPs; cardiovascular effects; coarse thoracic PM; fine PM; hepatic system effects; nervous system effects; PMx; pulmonary effects; ultrafine PM concentration*

Contents

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1. Introduction

Studying the health effects of ambient air pollution has been a challenging endeavor for environmental health scientists for many reasons. Epidemiologists have documented statistically significant associations between the routinely measured mass concentrations of particulate matter (PM) and excess mortality, morbidity, lost function, and lost time at work or school, and these statistically significant associations are usually stronger than those with routinely measured pollutant gases (US EPA, 2004, 2008). Although the relative risks (RRs) for mortality and nonscheduled hospital admissions are small, requiring sophisticated mathematical models for analysis in epidemiological studies, the populations studied and the and the populations at risk are quite large (Pope et al., 2009; Schwartz et al, 2008; Eftim et al., 2008; Miller et al., 2007), resulting in potentially very large public health impacts (e.g. thousands of cases annually in the United States). The bulk of this risk appears to be borne by the elderly or those in poor health, or both.

It seems highly unlikely that the effects are caused by nonspecific PM mass. Rather, it is likely that some specific chemical components within the PM mixtures are more potent than other components. The situation is complicated by the fact that PM is present in the air over a broad range of chemical compositions and particle sizes. Coarse dust particles with aerodynamic diameters above 10 μm do not normally penetrate beyond the larynx, have not been associated with health effects due to routine air pollution exposures, and are not routinely monitored. Particles with aerodynamic diameters below 10 μm, known as PM_{10}, can deposit along the conductive airways in the thorax, and nearly all of those with aerodynamic diameters below 2.5 μm penetrate into the gas-exchange region where particle retention times are much larger than for those that deposit on the conductive airways. A mucociliary blanket covering the conductive airways facilitates fairly rapid particle removal to the gastrointestinal tract. Furthermore, the smaller particles, known as fine PM or $PM_{2.5}$, are chemical mixtures that are quite different from the larger ones. The larger particles are mostly mineral in composition, whereas the $PM_{2.5}$ is composed largely of primary emissions of diesel engine soot particles and secondary aerosol formed by chemical transformations in the atmosphere from fossil fuel combustion products (both inorganic and organic vapors) and organic vapors from natural biologic processes. Most of these particles initially form as ultrafine PM (UFP), but rapidly aggregate into accumulation mode PM in the 0.1–2.5-μm size range. Suspicion concerning adverse health effects has centered on both fossil fuel combustion products and on inorganic compounds containing metals. Most of the mass of these metals is within the $PM_{2.5}$. A focus has often been on transition metals, such as iron (Fe), vanadium (V), nickel (Ni), chromium (Cr), copper (Cu), and zinc (Zn), or on carbonaceous compounds, on the basis of their ability to generate reactive oxygen species (ROS) in biological tissues. Most of the evidence pointing to the biological effects of metals, elemental carbon (EC), and organic carbon (OC) has come from studies involving exposures of laboratory animals *in vivo*, or of cells *in vitro*. We know of no studies involving exposures of laboratory animals *in vivo*, or of cells

in vitro to pure chemicals and their compounds, at doses with environmental relevance, that have been positive. On the other hand, some toxicological studies using high PM mass exposures to diluted tailpipe emissions, especially whole diesel engine exhaust (WDE), or to source-related PM mixtures containing multiple metals, such as residual oil fly ash (ROFA) or coal fly ash (CFA), and to concentrated ambient air particles (CAPs) have produced effects that appear to be related to their relatively low contents of metals and carbonaceous material. However, it has been difficult to determine the roles played by the individual components in the effects observed. Also, many laboratory-based studies have used resuspended dusts at relatively high mass concentrations, and the relevance of the effects observed to human ambient air exposures at much lower PM mass levels is therefore uncertain. Although effects found in high-dose laboratory *in vitro* exposures have occasionally been suggested to also occur with exposures of ambient air mixtures (e.g., inflammatory indicators in the *in vitro* exposures to CAPs in the study of Maciejczyk and Chen, 2005), more often effects have not been found (e.g., no abnormal levels of cytokines in human volunteers in the CAPs exposure study of Ghio et al, 2000a).

Studies of the effects of relatively low concentrations of airborne PM components in humans have all involved complex mixtures, and are one focus of this critical review. These include those short-term inhalation exposures to (1) CAPs in healthy human volunteers and (2) to diluted WDE, and (3) natural exposures to ambient PM, where data from simultaneous daily and/or seasonal or annual average PM compositional analyses were available for time-series and cross-sectional studies of effects in large human populations. Due to the limitations of statistical power in such natural population studies, the epidemiological analyses have focused more on identifying the contributions to the effects of factors or source-related mixtures than of individual components within the mixtures. Additional information comes from laboratory studies that have involved instillation of particle suspensions into human lungs and subsequent analyses of bronchoalveolar lavage fluid (BALF) samples for particle retention and biomarkers of effects.

Studies of the effects of relatively low concentrations of airborne PM components in laboratory animals that involve complex mixtures are another focus of this critical review. These include (1) short-term inhalation exposures to CAPs in mice, rats, and dogs; (2) subchronic inhalation exposures of CAPs to mice and rats; and (3) inhalation and intratracheal lung instillation of components and source-related mixtures.

A major objective of this critical review is to combine the analyses of the experimental studies with CAPs, and other ambient air PM components, in humans and other animals, with the associations between ambient air concentrations of PM and its components, to determine the nature and extent of the effects of ambient air PM and its components of major organ systems and their cross-species consistency, and to identify, as possible, the more potent PM components.

It is important to remember that all three particle size ranges are chemically nonspecific pollutant classes, and may originate from, or been derived from, various emission source types. Thus, PM toxicity may well vary, depending on its size distribution, source, and chemical composition. If the PM toxicity could be associated with specific source signatures, then health effects research could be better focused on specific PM components that come from those sources, and specific biological mechanisms could be postulated for further consideration by toxicological studies. PM health effects research is therefore now being increasingly focused on source-apportionment of PM using chemical speciation data, and this review of the CAPs literature emphasizes those CAPs studies that used PM compositional data to identify associations of exposures to PM source categories, or to individual PM components that have been associated with health-related effects.

In addition to this Introduction, this critical review paper consists of sections discussing: (2) Development of methods for conducting CAPs inhalation studies, and the advantages and limitations of the available technologies; (3) Studies in humans; (4) Studies in laboratory animals and *in vitro*; (5) Organ system responses; (6) Concordance of responses to CAPs and other exposures; (7) Summary of the role and contributions of CAPs studies; and (8) Unresolved issues and conclusions.

2. Development of methods for conducting CAPs inhalation studies

Laboratory investigators have recognized the need to do studies of health effects attributable to PM air pollution corresponding to those occurring in susceptible groups within natural populations. To do so, they need exposure atmospheres that faithfully represent what people actually breathe, while at the same time exposing them to a sufficient concentration to elicit measurable biological responses. Conducting such studies is inherently challenging because of (1) the extensive temporal and spatial variations in PM composition and particle size distribution; and (2) the lack of knowledge of which air pollutant components are most likely to be influential in causing the observed effects and their temporal patterns. Furthermore, the real-world exposures generally are not "square-wave" exposures as in conventional laboratory-based inhalation exposure studies, but rather are temporally and spatially variable. In addition, there are other variables among human populations, especially biological variables, whose influence on outcome measures can be great: for example, large variations in susceptibility due to age, genetic predisposition, diet, prior exposure, and disease history, as well as ventilation patterns and breathing rates during exposure can influence PM dosimetry. In addition, for many of the health-related measurements, it is not clear when, or with what frequency, they should be made. Finally, because the responses to ambient concentrations of PM are likely to be subtle, or to only occur in small

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