

Innovative Methods of Monitoring of Diesel Engine Exhaust Toxicity in Real Urban Traffic (MEDETOX)





Dear readers,

This monograph summarizes major results and findings of the LIFE project MEDETOX – “Innovative Methods of Monitoring of Diesel Engine Exhaust Toxicity in Real Urban Traffic”. The primary objective of the project was 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 the 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 project further disseminates the methodologies to relevant government and national / international regulatory authorities and other potential users (Ministry of Health/National Institute of Health, Municipal Council of Prague and councils of other big cities and their building authorities on the different level, OECD, CONCAWE, International Energy Agency (IEA), EC DG SANCO, EC DG Industry).

We identified, described and demonstrated the use of the standardized tests of toxicity as appropriate tool for regulatory and other environment policy decisions.

Last but not least we built effective interdisciplinary network targeted at holistic assessment of health risk potential of engine exhaust during real-world operation of road vehicles and mobile machinery. This was accomplished by a well-balanced team of experts on engines and emissions (Technical University of Liberec), toxicity assessment (Institute of Experimental Medicine of the Czech Academy of Sciences) and public policy (Ministry of the Environment of the Czech Republic).

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Coordinator of the Project MEDETOX

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1. Introduction

1.1. Particles emitted by internal combustion engines



Internal combustion engines, powering the majority of on-road vehicles, ships, and mobile machinery, are one of the principal sources of air pollution in most urban areas. Of these, ultrafine particles are of highest concern, followed by nitrogen oxides (NO_x).

The particles originate primarily from incomplete combustion of fuel and engine lubricating oil, and – at least in case of on-road vehicles, to a lesser extent – from uncombustible portion of fuel or lubricating oil and from engine wear. Primary particles emitted by engines, regardless of the fuel, are predominantly in the tens of nanometers (nm) in diameter [Kittelson, 1998]. Particles this small readily deposit in lungs [Gerde et al., 2001; Gehr and Rothen-Rutishauer, 2006]. Particles in lower tens of nm and smaller can penetrate through cell membranes into the blood, and have a wide and detrimental effect on human health [Künzli et al., 2000]. Diesel exhaust as well as air pollution were categorized by International Agency for Research on Cancer (IARC) as carcinogenic to humans [IARC 2012 and 2013]. Proximity to sources of internal combustion engine exhaust has been associated with increased risks of asthma and other pulmonary diseases, heart attack, and other chronic health problems [Lewtas, 2007; McEntee and Ogneva-Himmelberger, 2008; Balmes et al., 2009]. Lwebuga-Mukasa (2004) have reported that asthma prevalence was found to be correlated to truck traffic volumes.

Ultrafine particles (UFP) are often considered to be more hazardous to health than “average” particulate matter. UFP exposure has been already associated with some adverse health effects involving respiratory and cardiovascular system in the humans [Peters et al., 1997; Politis and Lekkas, 2008; Devlin et al., 2014; Lanzinger et al., 2015]. There is also evidence that urban UFP, in comparison with larger particles, induced greater systemic oxidative stress in the rat which was enhanced by an inhibition of the anti-inflammatory capacity and subsequently led to larger atherosclerotic lesions [Araujo et al., 2008]. Epidemiological studies have found association between residence location near the busy road and pulmonary impairments [Nuvolone et al., 2011; Lindgren et al., 2009].

Nitrogen oxides (NO_x), the sum of nitrogen monoxide (NO) and dioxide (NO_2), originate from atmospheric nitrogen during high temperatures and pressures inside the combustion chamber. The combustion process produces primarily NO. NO_2 forms through gradual oxidation of NO in the atmosphere. NO_2 can also be produced from NO by certain types of oxidation catalysts used primarily on diesel engines. NO_2 has a direct negative effect on human health, including low lung function and increased risk of cancer [Adam et al., 2015; Hamra et al., 2015; WHO, 2013].

1.2. Engine emissions and health effects

Engine emissions, or more precisely, exhaust emissions from internal combustion engines, both of spark ignition (typically gasoline) and compression ignition (typically diesel) type, belong among the major anthropogenic sources of air pollution in urban areas. The magnitude of this problem was already mentioned in 1999 by the United States Environmental Protection Agency (USEPA). According to USEPA, 83 % of people living in the USA were known to be exposed to concentrated diesel emissions from sources such as highways, heavy industries, construction sites, bus and truck depots, etc. [USEPA, 1999].

Even though diesel engines produce 28 times less carbon monoxide than gasoline engines, diesel exhaust has been evaluated as a hundred times more toxic in comparison with gasoline exhaust. Moreover, diesel cars also emit around 20 times more nitrogen oxides than gasoline cars [Krivoshto et al., 2008]. Their general contribution to the overall quantity of particulate matter (PM) in ambient air in urban areas was estimated as higher than 50 % for the fraction of aerodynamic diameter of $10 \mu\text{m}$ and less (PM_{10}) [USEPA, 2002]. Production of diesel exhaust particles is accompanied by adsorption of organic and inorganic compounds from the combustion process and/or the adsorption of additional compounds. The chemical composition of particulate matter of an aerodynamic diameter of $2.5 \mu\text{m}$ and less ($\text{PM}_{2.5}$) from diesel exhaust consistently showed mainly elemental carbon (33–90 %), organic carbon (7–49 %), sulfates and nitrates (1–4 %), metals & elements (1–5 %) and a fraction of other chemicals (1–10 %) [Betha et al., 2012].

The overall impact of engine emissions on human health has been studied for a long time, mainly due to the presence of polycyclic aromatic hydrocarbons (PAHs) and their derivatives (nitro-PAHs) in diesel exhaust particles. In 1988, the International Agency for Research on Cancer (IARC) classified diesel engine exhaust as Group 2A (probably carcinogenic to humans), but in 2012, based on new evidence concerning the association with increased risk of lung cancer, it was reclassified as Group 1 (carcinogenic to humans). Moreover, exposure to diesel engine exhaust is also positively associated, but with limited evidence, with an increased risk of bladder cancer. Gasoline exhaust is also in the area of interest of the IARC; in 1989 it was classified as possibly carcinogenic to humans (Group 2B) [IARC, 2012; IARC, 1989].

The above mentioned facts indicate that exhaust from diesel vehicles has been regarded to be significantly more harmful than that from gasoline vehicles. Beside carcinogenicity to humans, engine emissions as a whole were also associated with an increased risk of other health problems [Lewtas, 2007]. Traffic-related air pollution has been shown as an established risk factor for triggering myocardial infarction [Bard et al., 2014], analysis of the causes of chronic cough revealed a relation to exposure to diesel exhaust, ozone, nitrogen oxides, sulphur oxides and other environmental factors [Groneberg-Kloft et al., 2006], exposure to diesel exhaust particles has also been associated with increased production of inflammatory cytokines in bronchial epithelial cells [Doornaert et al., 2003], neurobehavioral problems [Kilburn, 2000], and recently, long-time exposure to traffic-specific $PM_{2.5}$, as well as $PM_{2.5}$ from other sources was found to be positively related to increased blood pressure [Fuks et al., 2016]. Diesel exhaust exposure is further connected to decreased quality of sperm [Sram et al., 1999]. Prenatal exposure can lead to premature birth or low birth weight, congenital abnormalities and an increase in the mortality of infants [Ghosh et al., 2012].

Historically, emissions legislation pertaining to the type approval of new vehicles was the most effective measure to reduce vehicle emissions per km driven. While the emission limits were decreasing, the laboratory conditions to which the limits are applicable remained more or less the same. In the European Union (EU), the relative low power levels at which the engine is operating throughout the currently used NEDC driving cycle, the increasing sophistication of engines and exhaust aftertreatment and their control, the competitive nature of the automotive market, and the lack of regulatory oversight, has lead to a condition where the ratio of the emissions during real driving to the emissions measured during the type approval cycle is increasing.

On automobile diesel engines, high emissions of particulate matter (PM) and nitrogen oxides (NO_x) are associated primarily with high-power operation at higher speeds and loads than those encountered during the NEDC [Vojtisek-Lom et al., 2009; Weiss et al., 2012; Carslaw et al., 2011]. In many cases, high emissions of NO_x were observed even during regimes normally covered during the NEDC, due to deliberate manipulation of the engine controls by the manufacturers. A formal accusation of one specific manufacturer by the USEPA of such practice became known as “DieselGate”.

Real driving emissions measurements show that engine UFP emissions are not evenly distributed among vehicles or along the path of travel, but rather, a small number of vehicles is responsible for a large fraction of the total emissions from the vehicle fleet [Wang et al., 2011; Kumar et al., 2011], and a large fraction of UFP emissions from a given vehicle is attributable to short episodes with high emissions [Vojtisek-Lom et al., 2015].

Additional pollutants are becoming of concern with the introduction of new engine and exhaust aftertreatment technologies and new fuels. For example, the use of catalytic devices for reduction of NO_x has led to emissions of ammonia (NH_3) and greenhouse gas nitrous oxide (N_2O), the use of certain types of oxidation catalysts has increased the share of NO_2 in NO_x in primary exhaust, biofuels and oxygenated fuels have increased the emissions of carbonyls, mainly formaldehyde and acetaldehyde.

New technologies and fuels also have an effect on the structure, size and other physical properties of particles, and on the composition of the organic compounds both in gaseous and particle phases, including semi-volatile organic compounds absorbed in or adsorbed onto particles.

Large portion of the health effects of the particles is, however, associated with compounds representing only a very small fraction of the total mass, such as polycyclic aromatic hydrocarbons (PAHs). It is also known that the combined toxicological effect due to the interaction of various compounds cannot be expressed as a mere sum of the effects of the individual compounds. Rating of health risks should therefore be based primarily on interaction of exhaust components with model biological systems, and not based only on chemical analysis of its composition. For this reason, assessment of the effect of new engine and exhaust aftertreatment technologies and new fuels should include toxicity tests which would serve as a more direct metric of the resulting health effect.

2. Major aims of MEDETOX project

Construction and validation of several innovative emissions monitoring and / or sampling systems for assessment of emissions on the road.

Preparation and validation of simplified toxicity assays which could be carried on the collected samples.

Investigation of real-world emissions and of the toxicity of particle-bound organic compounds collected during real-world operation and during its simulation in the laboratory, with particular focus on urban driving and on the effects of congestion.

Investigation of real-world emissions and of the toxicity of particle-bound organic compounds collected during operation of engines on various candidate replacement fuels.

3. Emissions sampling and measuring systems demonstrated (MEDETOX Action 3)

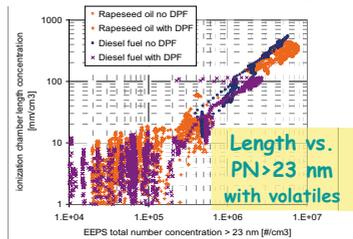
3.1. On-board emissions monitoring system

A miniature, low-cost portable, on-board exhaust emissions monitoring system designed by Vojtisek [Vojtisek-Lom and Cobb, 1998] was used as a primary monitoring system (*Figs. 1 and 2*). The system samples raw, undiluted exhaust gases via a 6 mm diameter stainless steel tube inserted into the tailpipe, and a 6 mm internal diameter, 5 m long conductive fuel line used as a sample line. The sample passes through condensation bowl where condensate is trapped and periodically removed. The sample is then reheated to approximately 60 °C by passing through a resistance-heated copper coil.



Portable on-board exhaust emissions monitoring system (current version)
HC, CO, NO, NO₂, PM mass, PM length

heated ionization "fire detector"
undiluted raw exhaust
 (multiplied by intake air flow for comparison measurements)
 ~ 0.1 mg/m³ sensitivity
 cheap (100 EUR)
 "poor man's PEMS" concept



Online real-world measurements
 Incl. PM on SI
 SAE 2013-24-0102
 SAE 2013-24-0168

Installs on motorcycles to locomotives
 This example → from a Euro 4 diesel car

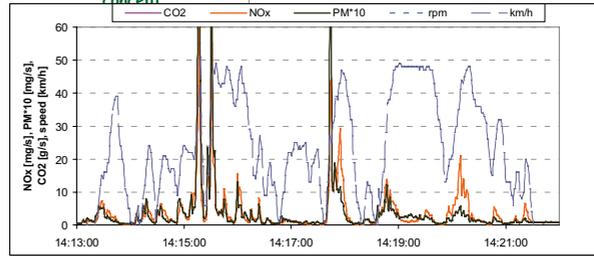


Fig. 1: Miniature PEMS with particle measuring capability.

On-board monitoring system

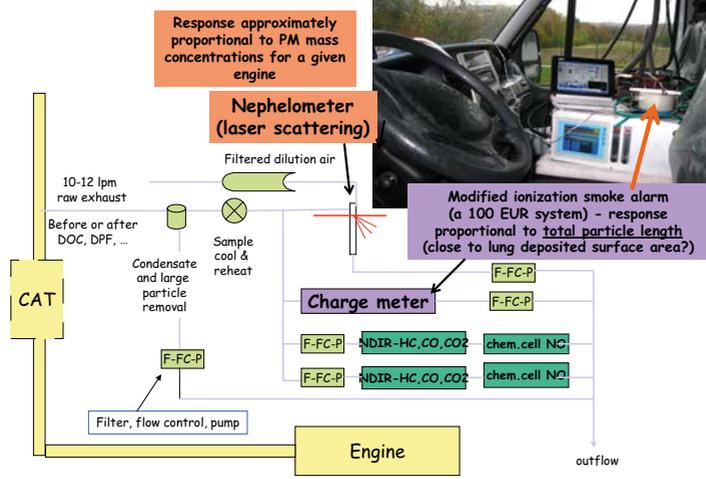


Fig. 2: On-board portable monitoring system schematics.

Concentrations of nitrogen monoxide (NO), carbon monoxide (CO) and carbon dioxide (CO₂) were measured online with a pair of modified, optimized and tuned BAR-97 grade analyzers, utilizing non-dispersive infra-red analyzers (HC, CO, CO₂) and electrochemical cells (NO). For most gasoline vehicles and for diesel vehicles without a diesel oxidation catalyst, the volumetric concentrations of total nitrogen oxides (NO_x) were assumed to be identical to those of NO. This assumption has been verified by extensive comparison tests of the on-board system, and is also in agreement with analogous sensors being used, in many regions, in periodic emissions inspections of spark ignition vehicles nominally operating at stoichiometric ratio. This is also in agreement with general experience that for engines with no catalytic devices and for engines operating mostly at stoichiometric conditions, the concentrations of nitrogen dioxide (NO₂) are several percent of the total nitrogen oxides (NO_x); the only engines known to produce relatively high emissions of NO₂ are those equipped with oxidation catalyst and operating lean (with excess air). At a later time, an electrochemical NO₂ sensor has been added to the apparatus.

Concentrations of particulate matter were measured online with a forward scattering integrating nephelometer, which, for a given engine and a given setup, tends to provide output proportional to particle mass concentration.

Concentrations of particulate matter expressed as total particle length were measured with a modified industrial building smoke detector equipped with a measuring ionization chamber utilizing a small radioactive source (²⁴¹Am, 30 kBq) to ionize the air. When voltage is applied to the electrodes in the chamber, a small ionization current flows through the chamber. Particles entering the chamber absorb the ions and decrease the ionization current. The detector was modified so that ionization current can be sensed directly and recorded by a data acquisition system. Laboratory comparison tests carried on engine exhaust [Vojtisek-Lom, 2011; Vojtisek-Lom 2013] have shown that the system provides a response proportional to total particle length concentration (i.e., ft of particles per cu.in., or m/cm³), that is, the sum of electric mobility diameters of all particles in a unit of volume.

To determine the intake air mass flow, this value can be obtained from the engine control unit's diagnostic interface, from a mass air flow sensor, or calculated from the engine displacement, engine volumetric efficiency data, and measured temperature and pressure of the intake air using the speed-density method.

All data were synchronized to adjust for measurement delay of the analyzers. Exhaust flow was calculated from the intake air mass flow and from the composition of the exhaust gases [Vojtisek-Lom and Cobb, 1998]. Instantaneous mass emissions were computed by multiplying corresponding values of exhaust flow and concentrations [Vojtisek-Lom and Cobb, 1998]. Instantaneous position and speed was recorded by a Global positioning system (GPS) receiver.

The instrument was powered by an external battery, in order not to affect the vehicle load, and to sustain measurements during engine starts. The instrument was warmed up for one hour prior to the tests.

3.2. Proportional sampling system

Working principle

The functional diagram of the system is given in [Fig. 3](#). Dilution air is drawn from the outside through a HEPA filter and is metered by a mass flow controller (MFC) to a miniature dilution tunnel located near the sampling point and connected with the exhaust system with a thermally insulated transfer line. A sample is drawn from the dilution tunnel through a sampling filter (typically a 47 mm diameter) and is metered by a second MFC. The second MFC operates at a constant flow rate, typically in the range of 20–50 grams of exhaust gas per minute. The flow through the first MFC, providing dilution air, is varied depending on the exhaust gas flow, so that the flow of the raw exhaust, determined as the difference between the flow of the sample and the flow of the dilution air, is proportional to the instantaneous flow of the exhaust from the engine. The practical realization of the latest dilution tunnel, designed by Michal Voráček as his bachelor's thesis work at TU Liberec [Voracek, 2013], is shown in [Fig. 4](#).

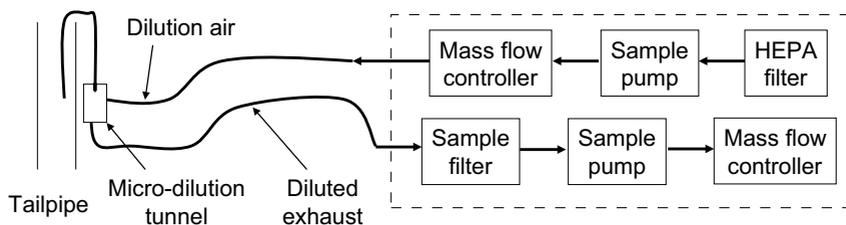


Fig. 3: Functional schematic of the sampling system.

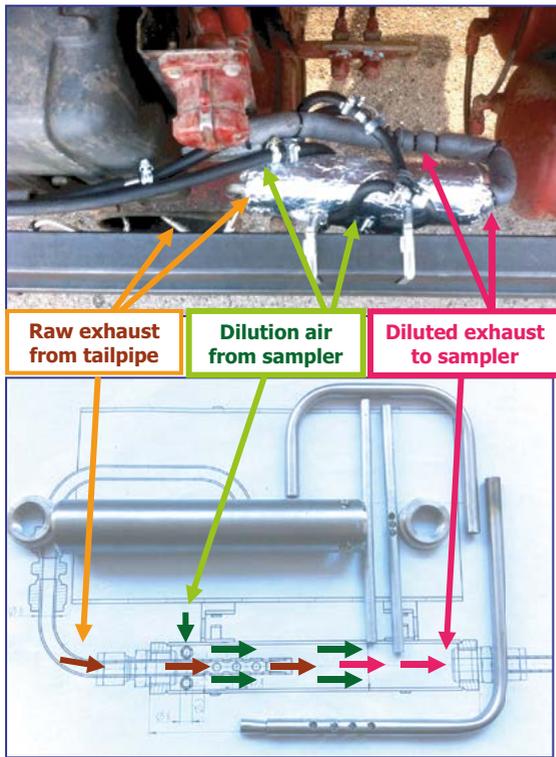


Fig. 4: Dilution tunnel as installed on Iveco Trakker heavy truck (photo on top), internal parts and drawing (bottom).

Dilution ratio and dilution air consideration

The dilution air flow is about 80 % to 90 % of the sample flow at the rated power, providing dilution ratios of 5:1 (80 %) to 10:1 (90 %), increasing to theoretically 100 % of the sample flow when the engine is stopped. In reality, when the engine is not running or the exhaust is not sampled, the dilution flow exceeds the sample flow, ensuring no exhaust entering the sample. The dilution ratio at full power is practically limited on the lower end to around 5:1 to prevent excessive formation of secondary aerosol, and, in more extreme cases, to prevent condensation of water vapor in the sampling line or on the filter. On the upper end, practical limits are imposed by the dilution ratio at lowest exhaust flow, typically at idle. If the maximum

exhaust flow is 5–10 times higher than the exhaust flow at idle, at 5:1 dilution ratio at maximum exhaust flow, the dilution ratio at idle is 25:1 to 50:1. At MFC accuracy of 1 %, the accuracy of exhaust flow is around 2 % of the dilution air flow, or, at 50:1 dilution ratio, around 100 % of the exhaust flow.

Exhaust flow determination

As the exhaust gas flow is rather difficult to measure directly due to its high temperature and content of particles, the flow of the dilution air is determined from the intake air flow, which is either measured by a mass flow meter, or, in case of on-board measurement, inferred from engine operating data. The intake air mass flow is obtained from the engine control unit via an EOBD interface, or computed from known engine displacement, known or assumed engine volumetric efficiency at given rpm and load, and engine rpm and intake air pressure and temperature using a formula

$$\text{MAF [g/s]} = 0,028967 * \text{nvol} * V [\text{dm}^3] * \text{rpm} * \text{MAP [kPa]} / \text{IAT [K]}$$

where nvol is engine volumetric efficiency at the given operating point, V is the engine displacement in liters, MAP is intake manifold absolute pressure in kPa, and IAT is the temperature of the charge in the intake air manifold in Kelvins. This formula is valid for the flow of the air through the engine; any recirculated exhaust must be subtracted. EGR rate can be obtained from the engine control unit, or determined experimentally, for example by measuring air-fuel ratio or concentrations of CO₂ simultaneously in the intake manifold and in the exhaust. This approach has been described in detail in an earlier work [Vojtisek-Lom and Cobb, 1998].

Transient response improvement

The mass flow controller used have a response time on the order of several seconds. To improve the transient response, without overly compromising the system stability, a fuzzy logic algorithm was implemented to alter the requested flow of dilution air when a transient change in engine intake air flow was detected. For example, during a rapid acceleration of the engine, the control system would decrease the flow of the dilution air so that the flow of the raw exhaust drawn into the diluted sample would increase in proportion to the increase in engine intake air flow. The fuzzy logic algorithm would then exaggerate the demanded change and request a considerably lower dilution air flow, in order to accelerate the transition to the new desired flow.

3.3. Comparison tests and reference systems

Several series of comparison tests were conducted on production and prototype diesel engines of various emission levels (Fig. 5); where non-production engines were used, no engine information is given. These tests were described in [Vojtisek and Pechout, 2013].

The aggregate results of all tests are shown in Fig. 6. As the duration of these tests spanned from minutes to tens of minutes, the results are reported on a grams per hour basis.

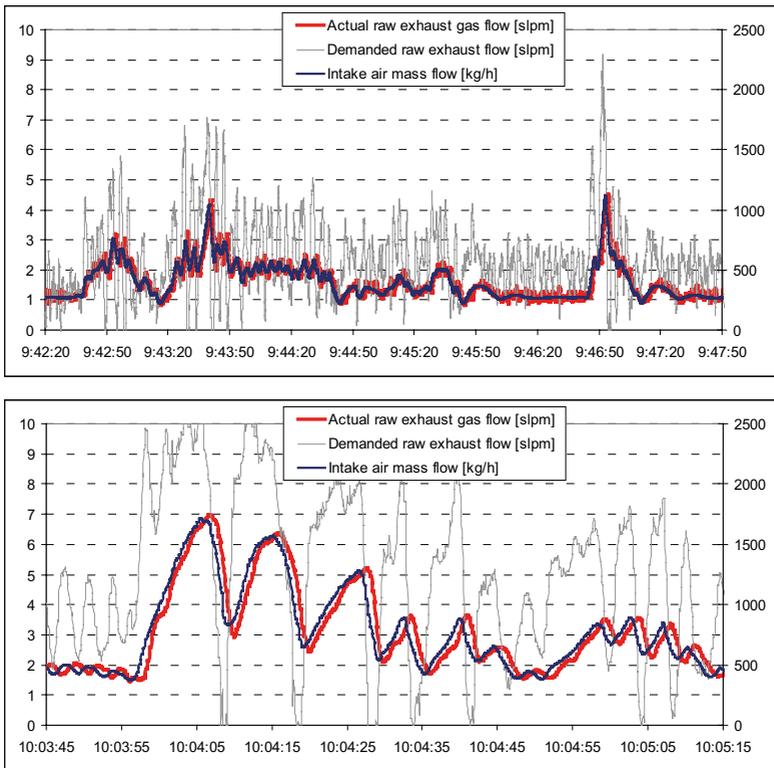


Fig. 5: Comparison of the actual raw exhaust gas flow into the sampling system with the actual engine intake air flow during three subsequent runs of the WHTC cycle.

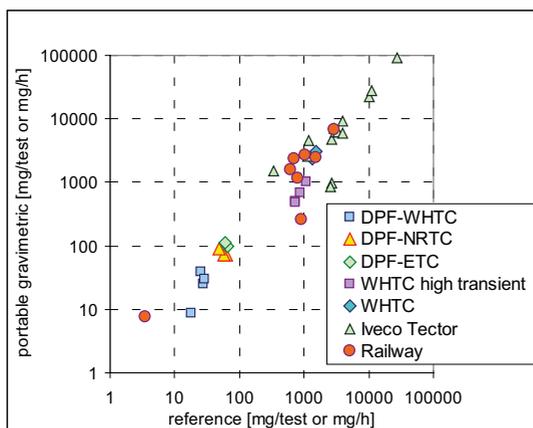


Fig. 6: Comparison of the PM mass emissions measured by the experimental system (vertical axis) against the respective reference measurements for all tests described here.

3.4. High-Volume Combustion Aerosol Sampling using Tandem Atmospheric Samplers

To facilitate the collection (dilution and sampling) of large quantities of particulate matter for detailed chemical analyses and for toxicological assays, a high-volume sampling system was designed.

A portion of raw exhaust was diluted at nominally 10:1 dilution ratio and sampled by a Hi-Vol sampler (EcoTech 3000) with a $PM_{2.5}$ impactor operating at $67.8 \text{ m}^3/\text{h}$ rate. The dilution air was provided by a second Hi-Vol sampler operating at 90 % of the nominal flow fed into a partial-flow dilution tunnel. Dilution ratio was verified by CO_2 concentrations measurements in raw exhaust and in the gases leaving the sampler. The actual dilution ratio was used for further computations.

Samples of PM were collected on $20 \times 25 \text{ cm}$ glass fiber fluorocarbon coated (Emfab, TX40HI20-VWW, Pall) and quartz fiber (QMA, Whatman) filters, with accumulations of ten to several hundreds of mg of PM per filter. The sampling setup is shown in Fig. 7. A total of three sampling sequences were run on each fuel, two with sampling on Emfab filters and one with sampling on QMA filters.

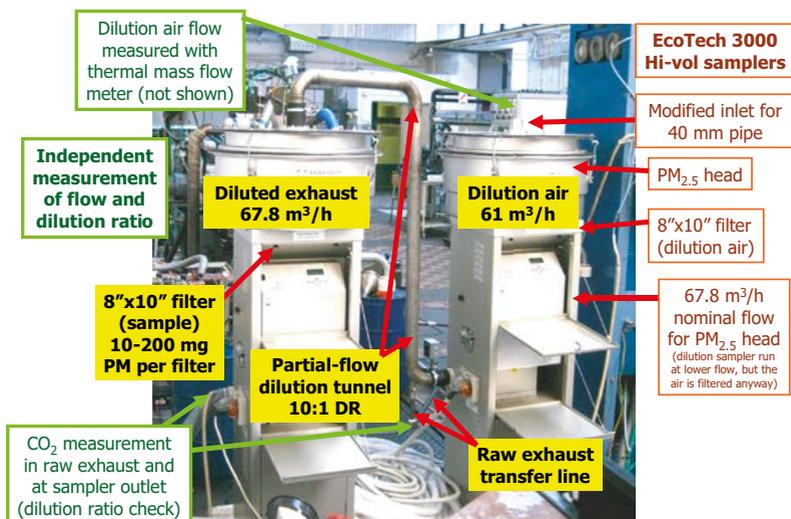


Fig. 7: Engine exhaust dilution and sampling system using a pair of modified high-volume atmospheric samplers.

To further increase the capacity of the samplers, they were later modified for use with diluted exhaust and subsequently augmented with an auxiliary three-stage blower to increase the effective filter capacity by approximately three times.

3.5. Portable on-board FTIR

Fourier Transform Infra Red (FTIR) spectrometers are used to collect infrared spectra (Fig. 8).

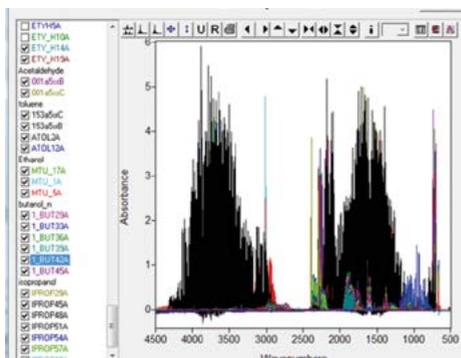
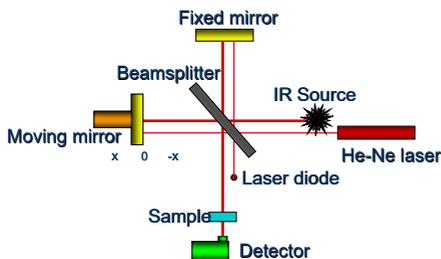


Fig.8: FTIR schematic (left, source: Nicolet), FTIR spectra of individual analyzed compounds (right, source: AutoQuant software, MIDAC).

The absorption spectra is the sum of the absorption spectra of individual compounds (Fig. 9). By deconvoluting the absorption spectra, the individual absorption spectra can be, at varying accuracy, obtained and used to calculate the concentrations of the pollutants of interest. FTIR allows for the detection of many compounds with heterogeneous molecules, including greenhouse gases CO_2 , CH_4 (methane) and N_2O (nitrous oxide), reactive nitrogen compounds such as NO , NO_2 , NH_3 , and hydrocarbons such as formaldehyde, acetaldehyde, n-alkanes, propene, 1,3-butadiene, and others.

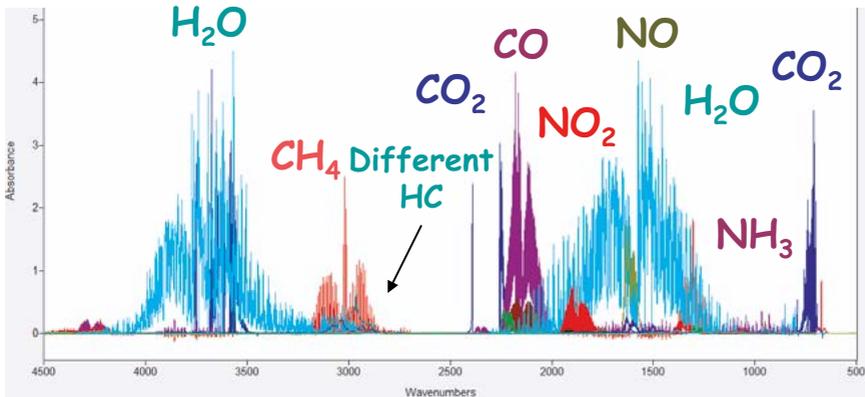


Fig. 9: Sample library spectra of principal compounds analyzed in engine exhaust.

Examples of use of the FTIR in MEDETOX prototype are shown in *Figs. 10 and 11*.

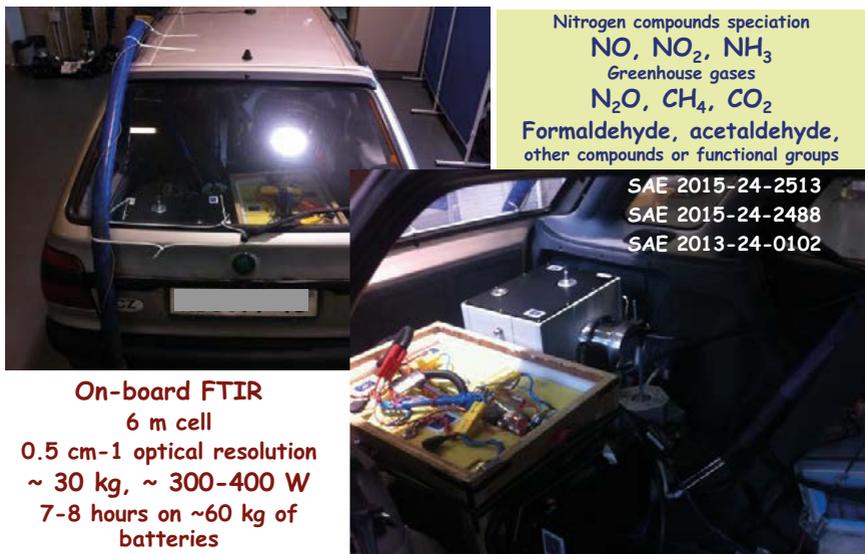


Fig. 10: FTIR system mounted on a older passenger car operated on alcohol fuels within a student research project [Vojtisek-Lom et al., 2013; Vojtisek-Lom et al., 2015; Pechout et al., 2015].

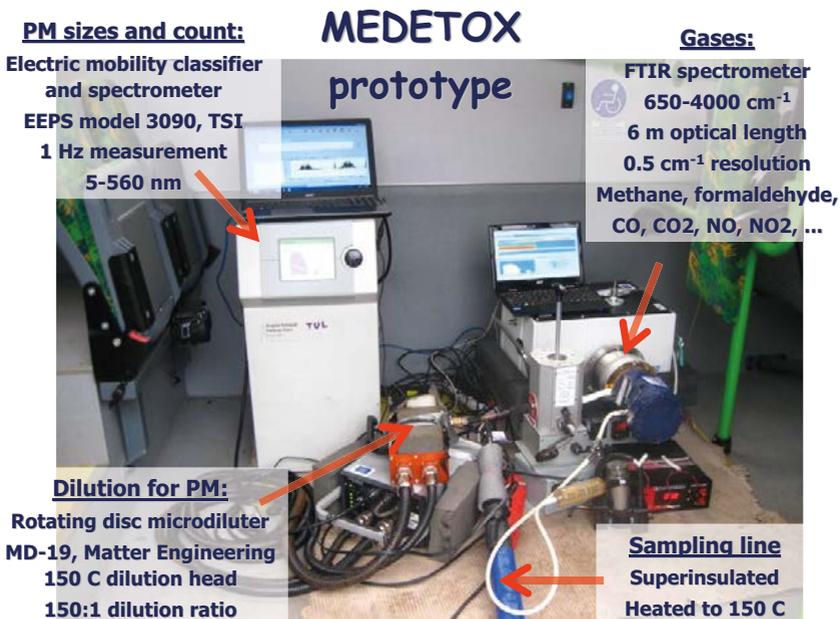


Fig. 11: FTIR as a part of a prototype for gaseous and particulate emissions measurements.

4. Toxicity tests optimization (MEDETOX Action 4)

4.1. Toxicity evaluation methods

All the methods of DEP toxicity testing are described as Standard operating procedures in details on MEDETOX website: <http://www.medetox.cz/methods/toxicity-evaluation>.

4.1.1. Cytotoxicity tests

Trypan blue cell viability test

The dye exclusion test is used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, Eosin, or propidium, whereas dead cells do not. In this test, a cell suspension is simply mixed with dye and then visually examined to determine whether cells take up or exclude dye. In the protocol presented here, a viable cell will have a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm.

WST-1 test for the quantification of cell proliferation, cell viability, and cytotoxicity

The stable tetrazolium salt WST-1 is cleaved to a soluble formazan by a complex cellular mechanism that occurs primarily at the cell surface. This bioreduction is largely dependent on the glycolytic production of NAD(P)H in viable cells. Therefore, the amount of formazan dye formed directly correlates to the number of metabolically active cells in the culture. Cells, grown in a 96-well tissue culture plate, are incubated with the ready-to-use WST-1 reagent for 2 hours at 37 °C. After this incubation period, the formazan dye formed is quantitated with a scanning multi-well spectrophotometer (ELISA reader). The measured absorbance at 440 nm directly correlates to the number of viable cells.

4.1.2. Analysis of DNA adducts of polycyclic aromatic hydrocarbons by ³²P-postlabeling

Carcinogen-modified DNA is digested enzymatically to deoxyribonucleoside 3'-monophosphates with endonuclease (micrococcal nuclease) and exonuclease (spleen phosphodiesterase). In order to increase the sensitivity of the method the enhancement procedure is used to enrich adducts. This procedure uses an enzymatic postincubation of DNA digests with nuclease P1 (from *Penicillium citrinum*). Nuclease P1 dephosphorylates deoxyribonucleoside 3'-monophosphates of normal nucleotides only to deoxyribonucleosides but not of adducted nucleotides. Deoxyribonucleosides do not serve as substrates of T4-polynucleotide kinase for the transfer of [³²P] phosphate from [γ -³²P]ATP. Then DNA hydrolysates are converted to 5'-³²P-labelled 3',5'-bisphosphates by incubation with [γ -³²P]ATP in the presence of carrier ATP and T4-polynucleotide kinase at pH 9.5. This alkaline pH is used in order to minimize the 3'-phosphatase activity of the polynucleotide kinase. ³²P-labelled adducts are separated and resolved from the excess of labelled non-modified nucleotides in two dimensions by multidirectional anion-exchange thin layer chromatography (TLC) on polyethyleneimine (PEI) cellulose plates (*Fig. 12*). During the first elutions (D1 and D2 directions) with aqueous electrolyte labelled unmodified nucleotides and

[³²P]-phosphate are removed from the origin for subsequent resolution using different solvent systems (D3, D4 directions). Location of adducts is carried out by screen enhanced autoradiography and visualized as dark distinct spots on X-ray films. These areas are then excised for quantitation by liquid scintillation. Adduct levels are calculated as relative adduct labelling (RAL) values, which represent the ratio of count rates of adducted nucleotides over count rates of total (adducted and normal) nucleotides. Utilizing the standard protocol, DNA adducts present at levels of 1 adduct in 10⁷ normal nucleotides (0.3 fmol adduct / μg DNA) can be detected.

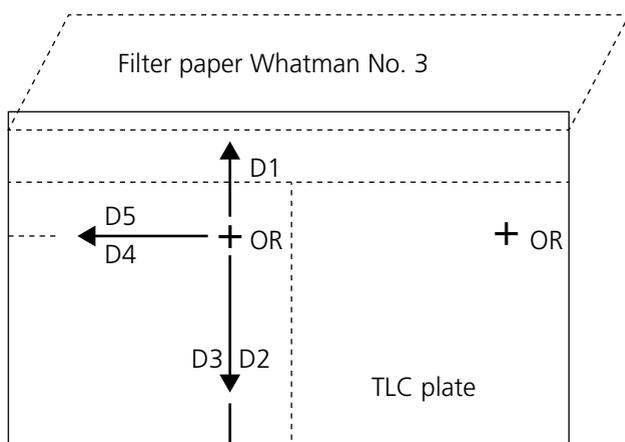


Fig. 12: Polyethyleneimine cellulose plate; OR: origin, D1–D5: directions of chromatography.

4.1.3. Oxidative damage of DNA, proteins and lipids in human lung cell lines

The methods to analyze oxidative damage of DNA, proteins and lipids by DEP in various human lung cell lines was standardized within Action 4 of project MEDETOX.

Oxidative damage of DNA

Method is based on the analysis of oxidized nucleotide 8-oxo-deoxyguanosine (8-OxodG). 8-OxodG levels were analyzed by competitive ELISA. Wells were coated with 5 ng of 8-OxoG conjugated with bovine serum albumin (BSA; total volume, 50 μ l/well) by drying the plates overnight at 37 °C. Plates were washed with PBS/Tween (0.05 % Tween 20 in PBS) and blocked with 200 μ l/well of blocking buffer (1 % FCS in PBS/Tween) for 1 h at 37 °C. After blocking, 50 μ l of 8-OxodG standards (concentration range, 1.25–40 ng/ml) and analyzed samples were added followed by 50 μ l of primary antibody (JalCA, Japan, clone N45.1, concentration 0.2 μ g/ml). After incubation for 1.5 h at 37 °C and washing, 100 μ l of secondary antibody conjugated with alkaline phosphatase (Sigma) were added. Another 1.5 h incubation at 37 °C was followed by washing with PBS/Tween and with 0.01 % diethanolamine in water. The color was developed by adding 100 μ l of p-nitrophenyl phosphate substrate (1 mg/ml of 1 mol/l diethanolamine) per well and incubating the plates for 30–60 min at 37 °C. The absorbance was measured with a microplate reader at 405 nm. Any samples with inhibition <20 % or >80 % were repeatedly analyzed either without dilution or with further dilution, respectively. Each sample was analyzed in triplicate. 8-OxodG concentration was expressed as nmol 8-OxodG/10⁵ dG.

Oxidative damage of proteins by DEP – protein carbonyl assay

The levels of protein carbonyl groups were assessed using a noncompetitive ELISA. The oxidized protein standards were prepared by incubation of BSA (50 mg/ml) with 0.73 M H₂O₂ and 0.42 mM Fe²⁺ for 1 h at 37 °C. The reaction was stopped with 40 μ M butylated hydroxytoluene. The carbonyl content of the oxidized BSA standard was measured spectrophotometrically. It was then diluted with native (unoxidized) BSA and PBS to give a final carbonyl content of 2.0 nmol/mg protein and protein concentration of 4 mg/ml. Total protein concentration in the samples was measured using Bicinchoninic Acid Kit (Sigma) and the samples were diluted with PBS to a final protein concentration of 4 mg/ml. After the derivatization with DNPH, the plate was coated with 200 μ l of sample and incubated overnight at 4 °C in the dark. The plate was washed with PBS/Tween (0.05 % Tween 20 in PBS) and blocked with 0.1 % BSA in PBS for 1.5 h. After another washing step, biotinylated primary anti-DNP antibody (Molecular Probes, OR, USA; diluted 1:1500 with 0.1 % BSA, 0.1 % Tween 20 in PBS) was added and the plate was incubated at 37 °C for 1 h. Another washing was followed by adding the streptavidin-biotinylated horseradish peroxidase conjugate (Amersham Biosciences, UK; diluted 1:4000 in 0.1 % BSA, 0.1 % Tween 20 in PBS)

and incubation at room temperature for 1 h. Color was developed by adding the tetramethyl benzidine (TMB) liquid substrate system (Sigma) and the reaction was stopped with H_2SO_4 after 15–25 min incubation in the dark. The absorbance was measured with a microplate reader at 450 nm. Each sample was analyzed in triplicate. Protein carbonyl concentration was expressed as nmol carbonyl/mg protein.

Lipid peroxidation by DEP – 15-F2t-Isoprostane immunoassay

15-F2t-IsoP levels were analyzed using immunoassay kits from Cayman Chemical Company (Ann Arbor, MI, USA). First, membrane-bound 15-F2t-IsoP was hydrolyzed and the samples were purified. Cell lysates containing 50 μg of protein were diluted with dH_2O to 100 μl . Then 100 μl of 15 % KOH was added and the samples were vortexed and incubated for 60 min at 40 °C. The pH of the samples was adjusted by adding 300 μl of 1 M KH_2PO_4 ; 100 μl of column buffer (containing 13.6 g KH_2PO_4 , 29.2 g NaCl, 0.5 g NaN_3 per 1000 ml, pH 7.4) was added, and the samples were mixed. The next step included adding 50 μl of Isoprostane Affinity Sorbent (Cayman Chemical Company) and incubating for 60 min at room temperature on a shaker. After the incubation, the samples were centrifuged for 1 min at 5,000 \times g and the supernatant was decanted. The 15-F2t-isoprostane bound to the sorbent was washed with 1 ml of dH_2O and eluted from the sorbent by re-suspension in 0.5 ml of elution solution (95 % ethanol). The samples were then stored in elution solution at -80°C until analysis. Before the assay, the samples were vacuum-dried, re-suspended in 110 μl of EIA buffer (supplied with the 15-F2t-IsoP kit) and immediately used for ELISA, which was performed according to the instructions provided by the manufacturer.

4.1.4. Genotoxicity induced by DEP – Comet assay

In the alkaline version of Single Cell Gel Electrophoresis (SCGE = Comet assay) the DNA is denaturated and next during the electrophoresis DNA migrates (as a polyanion) to anode. For visualization of DNA damage, observations are made of Ethidium Bromide stained DNA using a fluorescent microscope. A Comet assay analyser Lucia G software linked to a CCD camera is used to assess the quantitative and qualitative extent of DNA damage in the cells by measuring the length of DNA migration and the percentage of migrated DNA.

4.1.5. Chromosomal damage by DEP – Micronucleus test

The Micronucleus test is a comprehensive, quick and sensitive method for measuring DNA damage – micronuclei (MNI). Micronuclei are chromatin-containing structures in cytoplasm surrounded by a membrane without any detectable link to the cell nucleus. They are formed by exclusion of whole chromosomes or chromatin fragments during cell division. MNI are scored specifically in once divided binucleated cells. The micronuclei are biomarkers of chromosome breakage and/or whole chromosome loss.

4.2. Acellular and cellular systems for in vitro toxicity testing of engine emissions – advantages and limitations

In general, two basic test systems (acellular and cellular) are used in the genotoxicity testing of engine emissions. In acellular tests, the genotoxic effects are usually analyzed using calf thymus DNA (CT-DNA), optionally supplemented with microsomal S9 fraction that mimics the metabolic activation of the tested compounds in the cells. Thus, the genotoxic potential of organic extracts from particle emissions of diesel and rapeseed oil powered engines was analyzed using CT-DNA with a ³²P-postlabeling method. The results showed that PAH-DNA adduct levels depended on the type of engine and the test cycle rather than on the type of fuel [Topinka et al., 2012]. PAH levels in exhaust emissions from diesel engines powered by diesel and biodiesel fuels during extended low-load operation and the genotoxicity of the emissions were analyzed in another study using CT-DNA. There, the genotoxicity of extractable organic matter (EOM) from engine exhaust particles (measured as PAH-DNA adduct levels) was consistently higher for diesel than biodiesel, but oxidative damage induced by all tested EOMs was negligible [Vojtisek-Lom et al., 2015].

Even though acellular tests play an important role as the high-throughput and low-cost screening approaches for toxicity evaluation, cellular tests using cell lines represent a leading approach mainly due to species and tissue variety and better interpretation of the output in relation to living organisms. A huge variety of cell cultures are kept today mainly by: (i) the European Collection of Authenticated Cell Cultures (ECACC; <https://www.phe-culturecollections.org.uk>), the collection currently holds over 40,000 cell lines representing 45 different species, 50 tissue types, 300 HLA types, 450 monoclonal antibodies and at least 800 genetic disorders; and (ii) the American Type Culture Collection (ATCC; <https://www.lgcstandards-atcc.org/en>), that offers nearly 4,000 human cell lines that are invaluable for public health research, and a comprehensive selection of animal cell lines from over 150 different species. Some of these cell lines have already been used in various biomonitoring studies, including the testing of the cytotoxicity and genotoxicity of engine emissions. Despite the many advantages of cell lines it is also important to understand their limitations,

which are essentially given by the origin of each cell type. Cells or cell lines obtained from primary cultures can be considered the most suitable for genotoxicity tests. These cells represent original, non-tumor cells obtained from various tissues. Their potential for data interpretation is relatively high, but their main limitation is the fact that they stop dividing after a certain number of population doublings. Human peripheral lymphocytes, used in a lot of experiments, also fall into this category, but the limited amount of blood and variety of donors can be another limitation for inter-laboratory comparison of results. Immortalized cell lines, recruited either from naturally occurring cancer or as a result of induction of a viral gene that partially deregulates the cell cycle, seem to be a better solution to reduce the limits of cells senescence during cultivation. On the other hand, tests on a subgroup of immortalized cells, represented predominantly by cancer cells, have lower interpretation value due to the variety of karyotypes of these cells in cell culture, which can further increase during long-term cultivation [Watson et al., 2004]. Apart from the above-mentioned cellular systems, 3D tissues, containing various cell types and thus involving the interaction between cells and cell matrix, seem to be a promising novel model system for future experiments in this field of research. Due to their multicellular nature they lie on the borderline between standard cell cultures and complex organisms.

4.3. Biomarkers of biological effects of DEP

Although the selection of the cell line can be crucial for interpretation of the results, the methodological approach is also an inseparable part of experimental design. A battery of biomarkers, i.e. measurable indicators of a specific biological state, have been developed over the years for use as tools in evaluating the level of negative effects of various chemicals and mixtures, including emissions from various engines. Cytotoxicity assays are an indivisible part of genotoxicity tests using biomarkers, which should precede cellular tests with the aim to evaluate the maximum concentration of tested pollutants that are safe for the cells but ensure sufficient biological response to be detected on the biomarker level. Two main categories of biomarkers have been used in recent studies: (i) traditional biomarkers, and relatively new, (ii) –omics biomarkers using mainly molecular high-throughput approaches. The application of genotoxicity biomarkers associated with exposure to traffic and near-road atmosphere has been recently reviewed in human biomonitoring studies [DeMarini, 2013]. Traditional cytogenetic methods (including chromosome aberration (CAs), sister chromatid exchange (SCE) and analyses of micronuclei (MN)), detection of PAH-DNA adducts using the ³²P-postlabeling method, comet assay, oxidative stress analyses, including assessment of urinary 8-hydroxydeoxyguanosine, belong among the most frequently used methods. New –omics approaches mostly involve gene expression and DNA methylation studies that utilize both quantitative and qualitative methodological variants. From other methodological approaches, *HPRT* mutations, telomere length, DNA repair capacity or the effect of genetic polymorphisms are also analyzed across the studies.

The majority of these above-mentioned biomarkers can also be implemented in *in vitro* studies using various cell lines. In recent studies where traditional biomarkers were used, mammalian cell genotoxins in respirable diesel exhaust particles were identified using both comet and MN assays in human epithelial lung carcinoma cells A549 and Chinese hamster epithelial ovary CHO-K1 cells [Oh and Chung, 2006]. Increased MN formation and increased levels of specific DNA adducts was observed in human liver cancer HepG2 cells and in human B lymphoblastoid cell line MCL-5 treated with 3-nitrobenzanthrone (3-NBA) – a potent environmental mutagen found in diesel exhaust [Lamy et al., 2004; Arlt et al., 2004; Kawanishi et al., 2013]. Acute effects of diesel exhaust particles on oxidative stress were analyzed in cultured human kidney epithelial HEK 293 cells [Waly et al., 2013], effects of ultrafine petrol exhaust particles on cytotoxicity, oxidative stress induction, DNA damage and inflammation were observed in A549 cells and mouse RAW 264.7 macrophages [Durga et al., 2014], and the induction of lipid droplet formation in THP-1 derived macrophages was identified after exposure to automobile diesel exhaust particles [Cao et al., 2015]. The studies that utilized –omics biomarkers seem to be missing in this field of research and investigation of gene expression, microRNA expression or DNA methylation is prevalently limited to a few genes, microRNA or regions of DNA and did not utilize the methodological approaches to analyze a complete set of changes on the whole genome level.

Among the above-mentioned biomarkers, analyses of micronuclei belong to the most commonly used cytogenetic methods in genetic toxicology to assess chromosomal damage. Generally, this assay is applicable to a wide spectrum of biological materials and also offers a lot of technique variants, based on the purpose of the given study. The first analysis of MN was performed using plant biological material in 1959 [Evans et al., 1959]. A shift to cultivation of human cells [Elston, 1963] and the method improvement by inhibition of cytokinesis using cytochalasin-B in 1985 [Fenech and Morley, 1985] were other milestones in the methodological development. Current flexibility and multi-target genotoxic endpoints of the method offer its application in both *in vivo/ex vivo* and *in vitro* studies with possible application for a lot of mammalian cells, cell lines and 3D models [Fenech, 2007; OECD, 2014; Kirsch-Volders et al., 2011]. Also, both methodological variants: (i) with cytochalasin-B to analyze MN in binucleated cells (BNC) and/or, (ii) without cytochalasin-B for analysis of MN in mononuclear cells, are acceptable approaches based on the focus of the study and tested compounds.

As already mentioned, a variety of endpoints can be analyzed by micronucleus assay. Most of the laboratories assess DNA damage as a frequency of total MN using visual scoring, but “cytome” assay suggests a system in which every cell can be scored for its viability, mitotic status and chromosomal instability or damage status [Fenech, 2006]. Also, detailed detection of MN and the utilization of fluorescent staining can help to obtain important knowledge concerning the content and origin of MN (break or loss of the chromosome). Another effort to improve the method is the automation

of the scoring of cells with and without MN. Besides flow cytometry, analyzing MN using image cytometry currently represents a frequently used method. Various commercially available platforms of automated systems, their advantage, limitations and applicability for various biological material, including cell lines, was recently reviewed by users of these systems [Fenech et al., 2013].

Even though there is a relatively large number of biomonitoring studies focused on investigating the exposure to pollutants from traffic on human health by a wide spectrum of commonly used biomarkers, including the investigation of DNA damage using a micronucleus test in both peripheral lymphocytes and buccal cells [DeMarini, 2013], testing of the genotoxicity of individual emissions from fuels and their extracts from emissions after application of various operation modes of engines using various cell lines for investigating the frequency of MN are still scarce, even though the variety of cell lines suggested for this assay is relatively broad. Specifically, the OECD Guidelines for the Testing of Chemicals in *in vitro* mammalian cells using a micronucleus test suggest the use of cultured primary human or other mammalian peripheral blood lymphocytes and a number of human and rodent cell lines. Some limitations were suggested only for two human colon epithelial adenocarcinoma cells (HT29 and Caco-2), human hepatic progenitor cells HepaRG, HepG2, A549 and primary Syrian Hamster Embryo cells, which have been used for micronucleus testing but have not been extensively validated yet, and Mouse lymphoma cells (L5178Y), which are recommended to be used without cytochalasin-B due to the potential impact on cell grow. In addition, human peripheral blood lymphocytes should be obtained from young, non-smoking individuals with no known illness or recent exposures to genotoxic agents [OECD, 2014].

A detailed search of the PubMed database revealed that studies containing keywords: "micronuclei" and "engine emissions" and "cell line" are completely missing. Only a few relevant studies were found by various combinations of some of the following keywords: "micronuclei" or "chromosomal aberrations" or "DNA damage" and "engine emissions" or "diesel" or "biodiesel" or "diesel particulate extract" and "cell line". In total, six studies corresponding to the following criteria (testing engine emission extracts or their major compounds, utilizing cell lines or peripheral blood lymphocytes for *in vitro* testing and application of MN test) were selected [Oh and Chung, 2006; Lamy et al., 2004; Arlt et al, 2004; Gu et al., 2005; Bao et al., 2009; Odagiri et al., 1994]. From these studies, two used Chinese hamster cell lines V79 and CHO-K1 and analyzed the DNA damage level using a MN test. Diesel exhaust particulate matter substantially increased the frequency of MN in V79 lung cells beside a significant increase of CAs. Moreover, the results obtained from ovary CHO-K1 cells showed that the aromatic and slightly polar fraction of diesel exhaust particulate matter extracts induced chromosomal damage and DNA breakage in a non-cytotoxic dose [Oh and Chung, 2006; Gu et al., 2005]. Another study investigated the synergistic effects of the extracts of diesel exhaust particles and ultraviolet A radiation (UVA) in human-hamster hybrid (A₃) cells. The results, obtained also by MN test, provide

direct evidence of the augmented cytotoxicity and genotoxicity of diesel exhaust particles extracts activated by UVA [Bao et al., 2009]. Two studies tested the effect of 3-NBA, identified in diesel exhaust, in human HepG2 and MCL-5 cells. Comet assay and the ³²P-postlabeling method were also applied in these studies. Increased frequencies of MN as well as elevated DNA damage measured by other methods were consistently observed in both cell lines for the tested dose of 3-NBA [Lamy et al., 2004; Arlt et al., 2004]. Only one study used cultured human lymphocytes and an advanced variant of the MN test in combination with kinetochore labelling. The main results indicate that extracts from diesel emission particles, at least from light duty engines, preferentially induce whole chromosome loss (aneuploidy) rather than chromosome breaks [Odagiri et al., 1994].

Considering the above-mentioned knowledge, limitations and the lack of *in vitro* studies focused on testing the genotoxicity of engine emissions from biodiesel by micronucleus assay, we intended to fill this gap and suggested a plan for systematic testing of various types of engine emissions using an *in vitro* system, cell line and micronucleus assay. The effect of exposure to EOMs from diesel exhaust particles obtained from emissions of three types of fuels (B0 – neat diesel fuel, B30 – a blend of diesel fuel and 30 % biodiesel, B100 – neat biodiesel) and the major diesel components (benzo[a]pyrene (B[a]P), 3-nitrobenzanthrone (3-NBA), 1-nitropyrene (1-NP)) were tested using the immortalized, non-tumor human bronchial epithelial cells BEAS-2B and adapted MN test with optimized cultivation protocol.

5. Real Traffic and Biofuel Toxicity case studies (MEDETOX Actions 5–7)

5.1. Case study #1: Genotoxicity of diesel emissions in real world driving: Effects of cold starts, congestion, and DPF

Genotoxicity of organic extracts of particulate matter collected during real driving of two diesel trucks was evaluated in this study. A Euro 3 Iveco Trakker with no aftertreatment and a Euro 5 Iveco Daily truck with DOC+DPF were driven around Prague each for two days (Figs. 13–15). Exhaust emissions were measured by portable on-board emissions monitoring system. Particulate matter was sampled on Pall TX40HI20-WWW filters by a miniature on-board proportional sampling system.



Fig. 13: Sampling system mounted on a DAF Euro 5 truck with a SCR NO_x reduction catalyst.



**EURO 5 – DOC, DPF (particle filter), no SCR
2012 Iveco Daily, 3.0-liter Iveco engine**

**Emissions of particulate matter very low even during
1-hour idle and generally well below 1 mg/m³**

Fig. 14: Vehicle #1, Iveco Daily Euro 5 with a diesel particle filter (DPF).

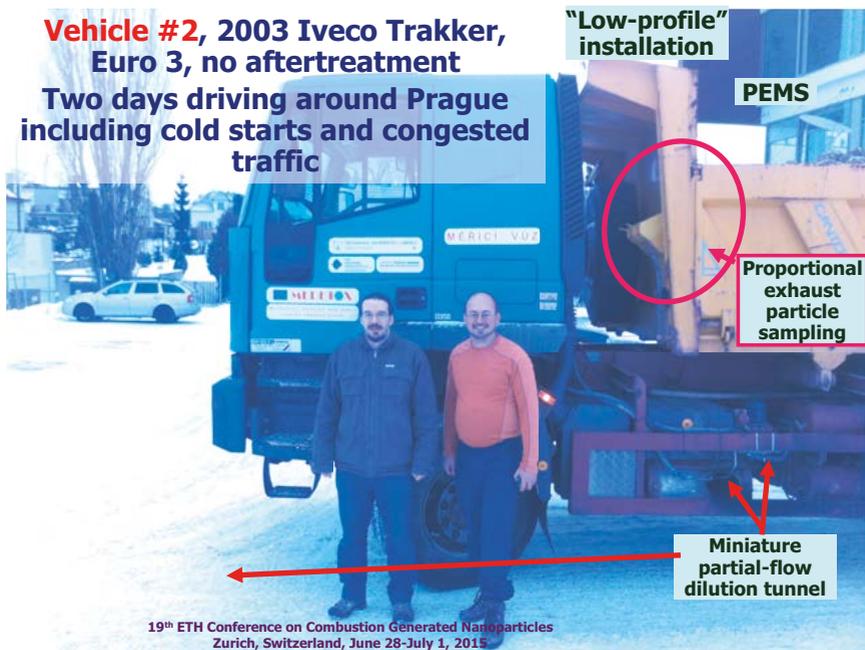


Fig. 15: Vehicle #2, Iveco Trakker, Euro 3, no exhaust aftertreatment.

Mean concentrations of particles in the exhaust and mean particle emissions per kg of fuel on the Iveco Trakker are shown in Fig. 16.

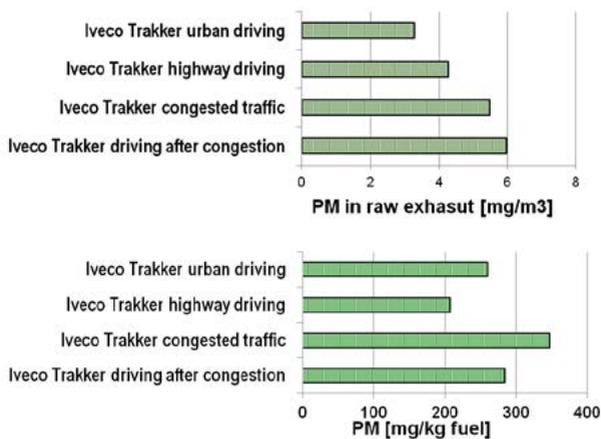


Fig. 16: Mean concentrations of particles in the exhaust and mean particle emissions per kg of fuel on the Iveco Trakker.

The filters were extracted by the mixture of dichloromethane/hexane (20:1 v/v). The aliquot of the extracts was used for chemical analysis of priority polycyclic aromatic hydrocarbons (PAHs), including 7 carcinogenic PAHs (cPAHs). The analysis of PAHs in exhaust of Iveco Trakker indicates significant concentrations of several cPAHs – benzo[a]pyrene, benz[a]anthracene and chrysene during cold idle, cold takeoff and cold city drive, while no PAHs were detected for any driving conditions in Iveco Daily Truck equipped with a diesel oxidation catalyst and a diesel particle filter (DOC+DPF). We further study genotoxicity (DNA adducts) and oxidative damage (8-oxo-deoxyguanosine, 8-oxo-dG) induced by organic compounds bound to exhaust particles. In agreement with cPAH content, the results show that on the engine without aftertreatment, genotoxicity of emissions was significantly elevated during cold start and cold operation of the engine.

Over the warmed-up phase, exposure to an equivalent of 10 dm³ of undiluted exhaust collected during congestion and during operation following the congestion has lead to more DNA adducts than “ordinary” urban and freeway operation (Fig. 17).

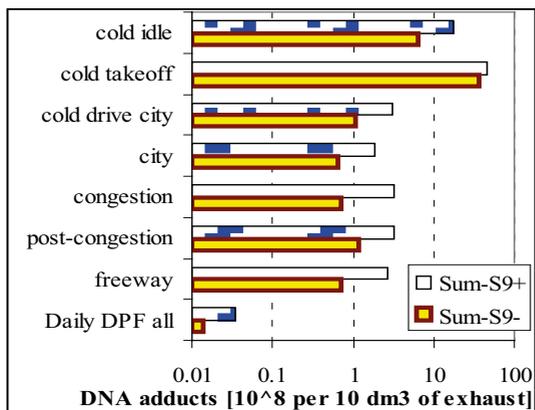


Fig. 17: Induction of DNA adduct levels in DNA treated with extractable organic matter (EOM) mass corresponding to 10 dm³ of undiluted engine exhaust for 24 h. Results for various engine regimens are shown for samples with metabolic activation of PAHs by rat liver microsomal fraction S9 (+S9) and without metabolic activation (-S9). The values represent the mean from two replicates varying by $\leq \pm 15\%$.

DNA adducts expressed per volume of raw exhaust and per kg of fuel consumed during warmed-up operation of the Iveco Trakker Euro 3 truck with no exhaust aftertreatment are shown in Fig. 18.

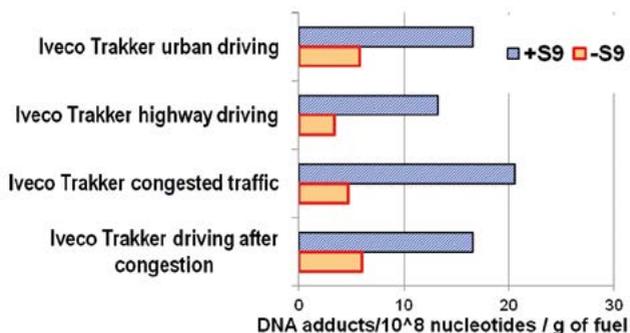
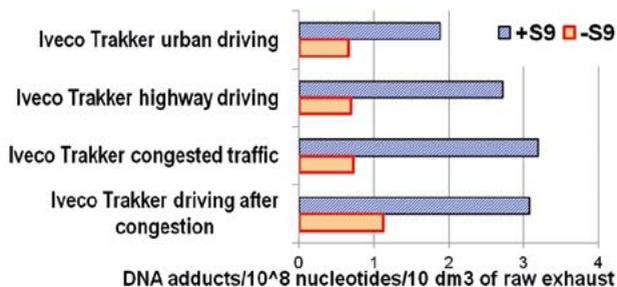


Fig. 18: DNA adducts expressed per volume of raw exhaust (top) and per kg of fuel consumed (bottom) during warmed-up operation of the Iveco Trakker Euro 3 truck with no exhaust aftertreatment.

Exhaust from the truck equipped with DOC+DPF had less DNA adducts, by approximately two orders of magnitude, compared to the truck without aftertreatment. In contrast to non-detectable PAHs in Iveco Daily truck emissions, we observed detectable DNA adducts induced by exhaust from this truck. This observation suggests high sensitivity of DNA adducts as a marker of genotoxic effects of engine emissions. The levels of 8-oxo-dG suggest weak effect of particle bound organic compounds on the extent of oxidative damage of DNA.

The results confirm that the benefits of DPF, observed in the reductions of particle mass and particle number emissions, also extend to the reduction of genotoxicity. The results suggest that cold start, cold operation and congestion increase the genotoxicity of exhaust emissions.

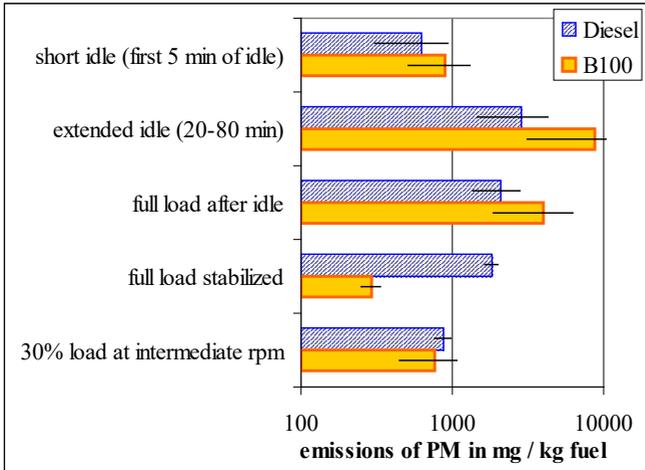
5.2. Case study #2: Simulation of extended idling in the laboratory: Polycyclic aromatic hydrocarbons (PAHs) and their genotoxicity in exhaust emissions from a diesel engine during extended low-load operation on diesel and biodiesel fuels

This case study investigates the effects of emissions including carcinogenic polycyclic aromatic hydrocarbons (cPAH) of a conventional diesel engine without a particle filter. Experiments were carried on during extended idle and during a loaded operation immediately following the extended idle. Extended low-load operation of diesel engines due to idling and creep at border crossings, loading areas and in severe congestion has been known to deteriorate the combustion and catalytic device performance and to increase the emissions of particulate matter (PM). A conventional diesel engine was coupled to a dynamometer and operated on diesel fuel and neat biodiesel alternately at idle speed and 2 % of rated power and at 30 % and 100 % load at intermediate speed. Exhaust was sampled on fiber filters, from which the content of elemental and organic carbon and polycyclic aromatic hydrocarbons (PAH), including cPAH and benzo[a]pyrene (B[a]P) have been determined. The emissions of cPAH and B[a]P have increased 4–6 times on diesel fuel and by 4–21 % on biodiesel during extended idling relative to a short idle and 8–12 times on diesel fuel and 2–20 times during subsequent operation at full load relative to stabilized operation at full load. The total “excess” cPAH emissions after the transition to full load were on the same order of magnitude as the total “excess” cPAH during extended idling. The absolute levels of PAH, cPAH and B[a]P emissions under all operating conditions were lower on biodiesel compared to diesel fuel. Genotoxicity of organic extracts of particles was analysed by acellular assay with calf thymus DNA (CT-DNA) and was consistently higher for diesel than for biodiesel. The exhaust generated during extended idle and subsequent full load exhibited the highest genotoxicity for both fuels. These two regimes are characterized by significant formation of cPAH as well as other DNA reactive compounds substantially contributing to the total genotoxicity. Oxidative DNA damage by all tested extracts was negligible.

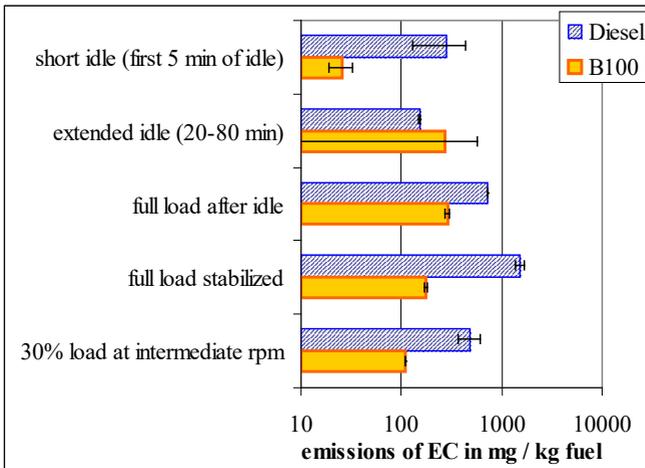
Major results of the study are depicted in [Figs. 19 and 20](#).

Fig. 19 A–E: Comparison of fuel-specific emissions of a) total PM mass, b) elemental carbon (EC), c) all PAH, d) carcinogenic PAH and e) benzo[a]pyrene across operating modes and between diesel fuel and neat biodiesel.

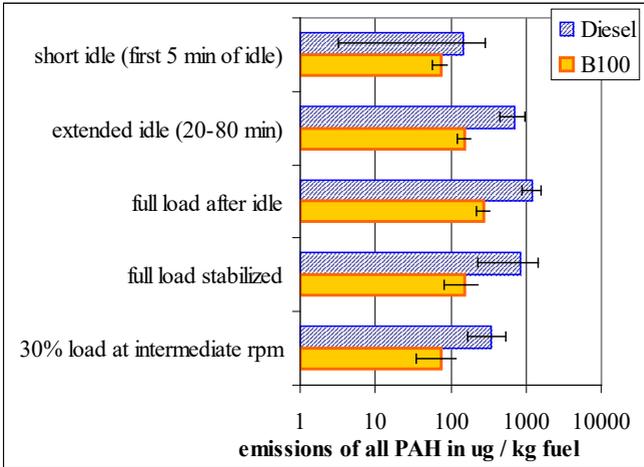
a)



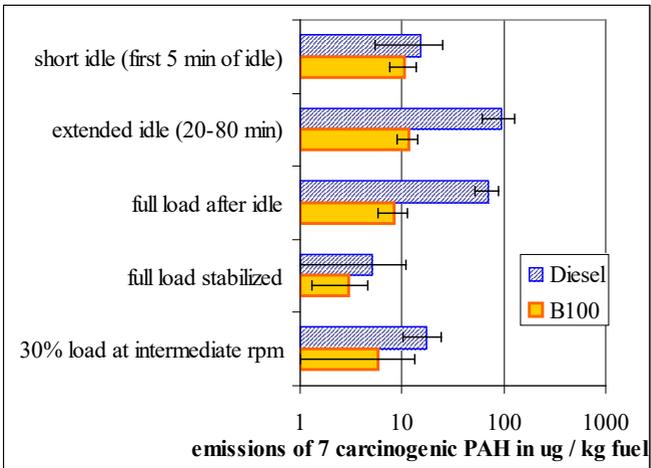
b)



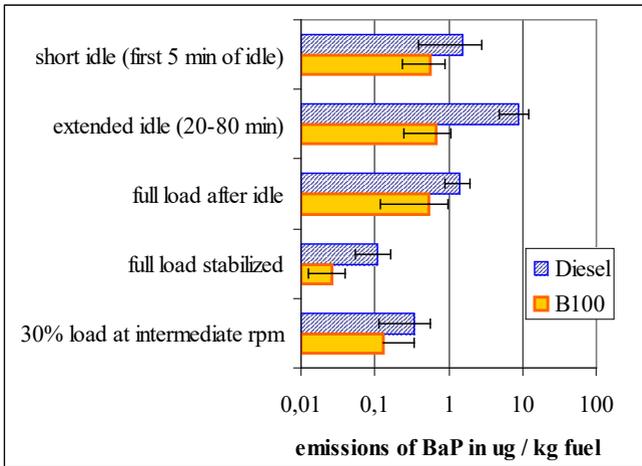
c)



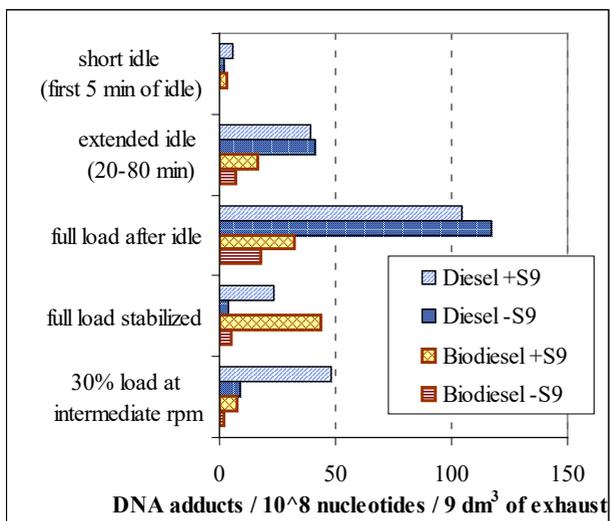
d)



e)



a)



b)

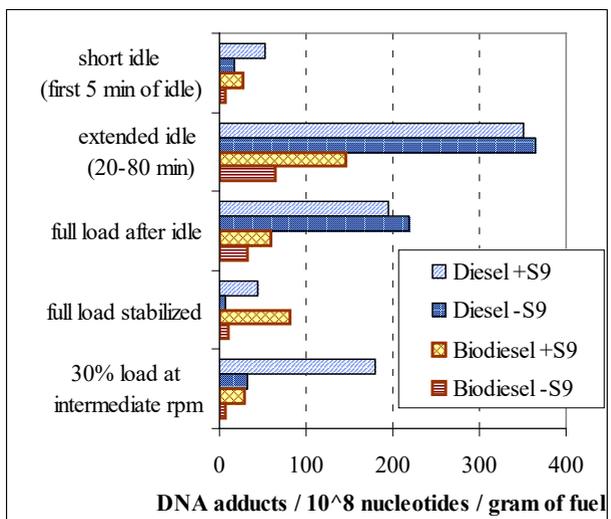


Fig. 20: Induction of DNA adduct levels in CT-DNA treated with extractable organic matter (EOM) corresponding to 9 dm³ of undiluted engine exhaust for 24 h. Results for diesel and biodiesel various operating modes are shown for samples with metabolic activation by rat liver microsomal fraction S9 (+S9) and without metabolic activation (-S9). DNA adduct levels per volume of undiluted exhaust (a) and gram of fuel (b) are shown. The values represent the mean from two replicates varying by $\pm 15\%$.

5.3. Case study #3: DNA damage potential of engine emissions measured in *in vitro* systems by micronucleus test

Internal combustion engine emissions belong among the major anthropogenic sources of air pollution in urban areas. Recently, the International Agency for Research on Cancer (IARC) concluded that there was sufficient evidence of the carcinogenicity of diesel exhaust in humans. Lately, alternative fuels, mainly biodiesel, have become popular. However, little is still known about the genotoxicity of emissions from these alternative fuels. Even though many human biomonitoring studies analyzing the potential harmful effects of engine emissions by application of various biomarkers were published, this MiniReview revises the knowledge obtained by *in vitro* studies with the aim of emphasizing the possibility to investigate DNA damage expressed as the frequency of micronuclei (MN) in cell lines. This intention is supported by a new study, in which we analyzed MN in human bronchial epithelial cells (BEAS-2B), induced by organic extracts from extractable organic matter (EOM) obtained from particle emissions from various blends of biodiesel with diesel fuels, and by major diesel exhaust organic/genotoxic components [benzo[a]pyrene (B[a]P), 1-nitropyrene (1-NP), 3-nitrobenzanthrone (3-NBA)]. The results of this study show that the genotoxicity of EOMs from the engine emissions of diesel and biodiesel engines are comparable. Moreover, both nitro-PAHs compounds demonstrated higher genotoxic potential in comparison with B[a]P. Challenges for future research, including testing of a variety of fuels, using broad variants of micronucleus assays, recommendations for cell line selection and application of -omics techniques with the aim to better understand the mechanism of action are discussed.

Engine emissions collection

Diesel particulate matter samples were collected at a transient engine dynamometer facility on an Iveco Tector heavy-duty diesel engine running a World Harmonized Transient Test Cycle (WHTC). Petroleum diesel fuel without a biocomponent (B0) and biodiesel (methyl esters of rapeseed oil) in its neat form (B100) and at 30% by volume blend with diesel fuel (B30) were used as test fuels. Diluted exhaust was sampled from a full flow dilution tunnel and constant volume sampler (CVS) by a pair

of modified atmospheric high-volume samplers (EcoTech 3000, 68 m³/h each) on fluorocarbon-coated glass fiber filters (Pall TX40HI20-WW), which were then extracted by dichloromethane and the extract was dissolved in dimethyl sulfoxide (DMSO) at a sample concentration of 0.1 g/ml DMSO.

Cell line description

Human bronchial epithelial cells BEAS-2B obtained from ATCC® (CRL9609™) were used to evaluate the genotoxic effect of both (i) EOMs and (ii) selected major diesel compounds. The BEAS-2B cells represent an adherent cell line derived from lung autopsy of a healthy man in 1988 [Reddel et al., 1988]. The cells are non-tumor, immortalized by hybrid Ad12-SV40 virus with typical epithelial morphology and metabolism. The cells are pseudodiploid and stable under conditions defined by ATCC.

Cell cultivation and treatment conditions

The protocol recommended by ATCC for cultivation of the BEAS-2B cells was used in this experiment in order to ensure the reproducibility of the experiments and stability of the cell line. Briefly, cultivation surfaces were coated with a mixture of 0.01 mg/ml fibronectin (Sigma-Aldrich), 0.03 mg/ml bovine collagen type I (Sigma-Aldrich) and 0.01 mg/ml bovine serum albumin (BSA) (Sigma-Aldrich) dissolved in bronchial epithelial basal medium (BEBM™) (Lonza) and kept in a 37 °C incubator overnight. Before seeding the cells, all the coating media was removed. Serum-free cultivation conditions (BEGM™ kit CC3170) (Lonza) were used. For all experiments, the cells confluence did not exceed 70 % to avoid terminal squamous differentiation.

Two exposure times (28 h and 48 h) and three concentrations of EOMs and major diesel exhaust carcinogenic compounds were selected for testing in this experiment. The tested concentrations were as follows: 1 µg/ml, 10 µg/ml, 25 µg/ml for EOMs from engine particle emissions; 25 µM, 100 µM, 200 µM for B[a]P (Sigma-Aldrich); 1 µM, 5 µM, 10 µM for 3-NBA (Chiron AS) and 1 µM, 5 µM, 50 µM for 1-NP (Sigma-Aldrich). All experiments were done in triplicate using the same passage of the cells.

Micronuclei analysis

The genotoxicity of the tested compounds was determined using the cytokinesis-block micronucleus assay in the 8-well Lab-Tek™ Chamber Slide System where the cells were grown and treated, thus allowing us to reduce the consumption of media and the cost of the experiment. The co-treatment version of cytokinesis-block micronucleus assay with simultaneous treatment with compounds and cytochalasin-B (Sigma-Aldrich) (concentration 1 µg/ml) for 28 h was done according to previously described conditions [Fenech, 2007; OECD, 2014].

At the end of cultivation, the cells were treated with a hypotonic solution of KCl (0.075M, Sigma-Aldrich) and fixed with a mixture of methanol (Merck Millipore) and acetic acid (Penta) (3:1).

After fixation, the slides were dried and stained by 5 % Giemsa (Merck Millipore). Visual scoring using the Olympus BX41 microscope was performed to analyse the BNC cells in final magnification 1000x. A total of 3x 500 BNC per each tested compound were evaluated and the cytokinesis-block proliferation index (CBPI) was calculated to control for cell division. The aberrant cells were recorded using a Canon EOS600D camera. The results were expressed as a percentage of aberrant binucleated cells with micronuclei (% ABB). Examples of BEAS-2B cells in various stages of experiment (cultivation, microscopic analysis) growing in appropriate and inappropriate conditions are shown in [Fig. 21 A–D](#).

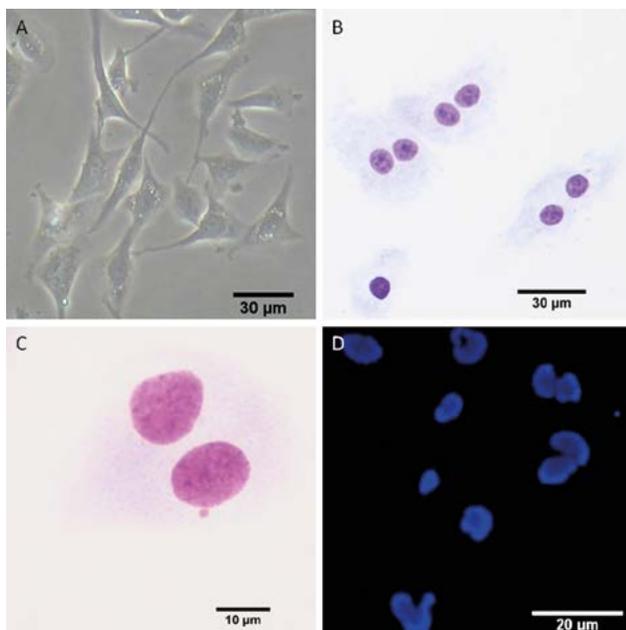


Fig. 21: Examples of BEAS-2B cell cultures: (A) BEAS-2B cells growing in Nunc® Lab-Tek® Chamber Slide™ System, visualized with phase contrast. (B) Giemsa-stained binucleated and mononucleated BEAS-2B cells fixed directly on cultivation surface. (C) Giemsa-stained binucleated BEAS-2B cell with micronuclei. (D) DAPI-stained nuclei of BEAS-2B cells growing in inappropriate medium, which caused morphological changes.

Statistical analysis

The two-proportion z-test to compare control groups vs. treated groups was used to analyze the data. The differences between groups were considered significant for $p < 0.05$.

Results

EOMs from diesel exhaust particles from all of the tested fuels (diesel, 30 % biodiesel and neat biodiesel) significantly increased the % ABB at the tested concentrations (Fig. 22 A–C).

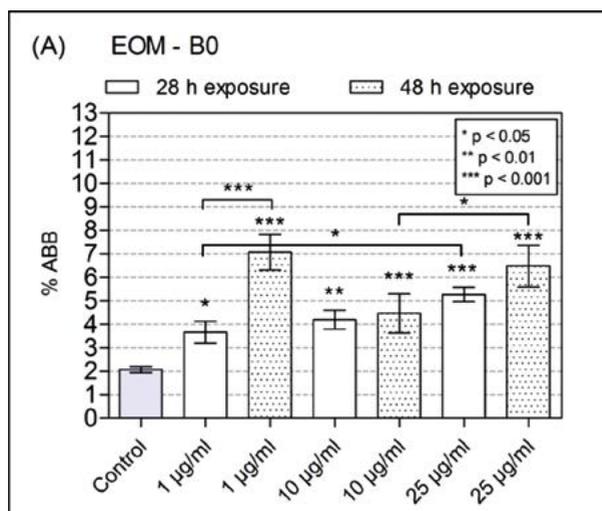


Fig. 22: The micronuclei formation after 28 h and 48 h exposure of BEAS-2B cell line to extractable organic matter (EOM) obtained from diesel emission particles of various types of fuels: (A) B0 – neat diesel fuel, (B) B30 – a blend of diesel fuel and 30 % biodiesel, (C) B100 – neat biodiesel. The genotoxic effect of tested compounds was measured as a percentage of aberrant binucleated cells with micronuclei (% ABB).

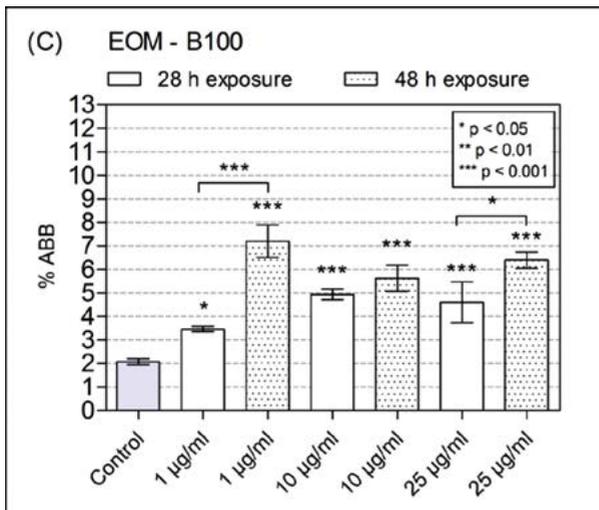
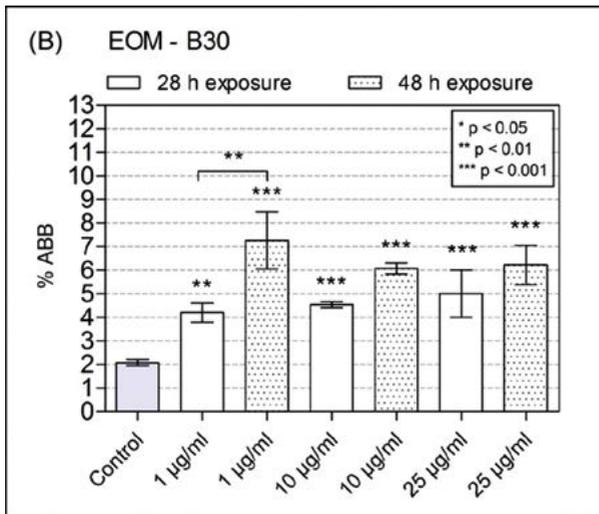


Fig. 22: continued.

B0 samples showed a good dose-dependent increase of the % ABB during 28 h of exposure, unlike 48 h exposure, where the lowest tested concentration (1 µg/ml) induced the highest increase of % ABB. For B30 samples, a dose-dependent increase of the % ABB was less obvious for 28 h treatment; the results for 48 h exposure had a trend similar to B0 samples. For B100 samples, we did not

see dose-dependent effects of either treatment. The exposure time significantly affected the % ABB at a concentration of 1 $\mu\text{g}/\text{ml}$ for all fuels and at a concentration 25 $\mu\text{g}/\text{ml}$ also for B100. Overall the % ABB was slightly higher after 48 h exposure than 28 h exposure.

To sum up, EOMs from the tested fuels showed a similar genotoxicity in BEAS-2B cells. A concentration of 1 $\mu\text{g}/\text{ml}$ of the tested EOMs after 48 h treatment was associated with the highest genotoxic effect (3.40x, 3.5x and 3.46x increase of % ABB in comparison with the control for B0, B30 and B100 EOM sample, respectively). In general, no significant differences were found between EOMs from the tested fuels.

Major diesel exhaust components (B[a]P, 3-NBA, 1-NP) mostly significantly increased the % ABB in BEAS-2B cells (Fig. 23).

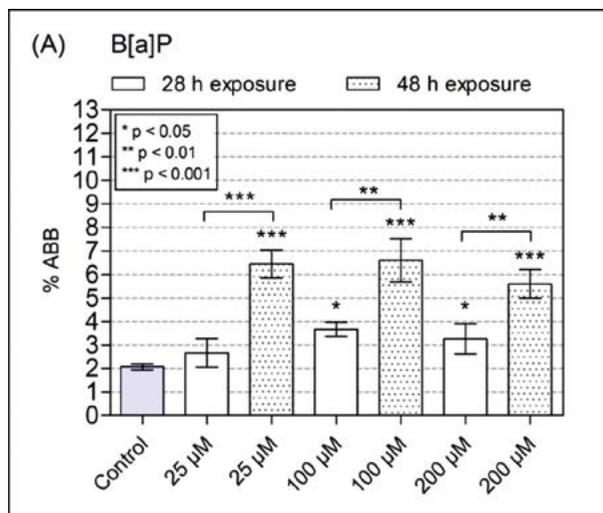


Fig. 23: (continued on the next page).

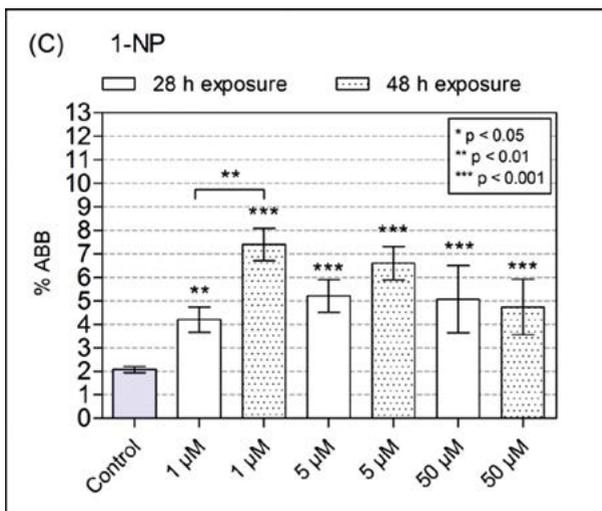
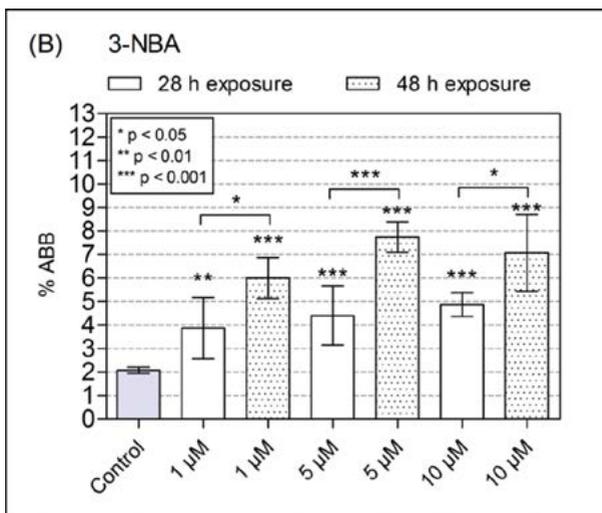


Fig. 23: The micronuclei formation after 28 h and 48 h exposure of BEAS-2B cell line to the major diesel emission components: (A) benzo[a]pyrene (B[a]P), (B) 3-nitrobenzanthrone (3-NBA), (C) 1-nitropyrene (1-NP). The genotoxic effect of tested compounds was measured as a percentage of aberrant binucleated cells with micronuclei (% ABB).

The only non-significant increase was observed for the 28 h treatment with 25 μ M B[a]P. For B[a]P exposure, no dose-dependent changes of the % ABB were found, but the effect of time of exposure was significant for all tested concentrations. Exposure to 3-NBA showed some dose-dependent changes for 28 h treatment and, similarly to B[a]P, a significant effect of time of exposure for the tested concentrations. The highest level of % ABB (7.73 %) was found after 48 h exposure to 5 μ M 3-NBA. Although the 1-NP treatment also significantly affected the % ABB, no apparent dose-dependent increase was detected for either exposure time. Interestingly, for 48 h treatment a dose-dependent decrease was observed. This finding could be associated with a decrease of the CBPI index up to the level of 1.55. The highest level of % ABB (7.4 %) was induced by the lowest dose of 1-NP (1 μ M) and 48 h of treatment.

All the tested PAHs had similar genotoxic effects, although nitro-PAHs induced a slightly higher % ABB than B[a]P after 28 h exposure.

In summary, our data indicate that both the major diesel exhaust components and EOMs from diesel and biodiesel fuels significantly increase the percentage of aberrant cells after both 28 and 48 h exposure, indicating their genotoxic potential in BEAS-2B cells. Interestingly, we did not find any differences in the genotoxicity of EOMs from exhaust of diesel and biodiesel fuels.

Discussion

The main intentions of this MiniReview were (i) to revise the knowledge obtained by *in vitro* studies focused on the potential harmful effects of engine emissions, with the aim to emphasize the possibility to investigate DNA damage using a micronucleus test in various cell lines; (ii) to support current knowledge by a new study and suggested optimal conditions for analyzing MN in non-tumor immortalized human bronchial epithelial cells after treatment with three EOMs from exhaust particles obtained from various fuels (B0 – neat diesel fuel; B30 – a blend of diesel fuel and 30 % biodiesel; and B100 – neat biodiesel) and three major diesel compounds (B[a]P, 3-NBA, 1-NP) and finally; (iii) to identify important gaps and challenges for future studies in this field of research.

Current research and new results

From the studies reviewed here, only six analyzed the potential harmful effect of engine emissions or their major compounds using the micronucleus test in a cell line [Oh and Chung, 2006; Lamy et al., 2004; Arlt et al., 2004; Gu et al., 2005; Bao et al., 2009; Odagiri et al., 1994]. All these studies demonstrated the ability of the MN assay to detect DNA damage after these treatments. However, from the cell lines used, only three were of human origin (tumor cell lines HepG2 and MCL-5 and peripheral blood lymphocytes), the other three were obtained from Chinese hamster. This clearly

demonstrates a lack of studies that use normal human cells. Further, these studies focused on the effects of model compounds and diesel exhaust particulate material or extracts, but none of them tested the impacts of biodiesel emissions or their extracts. This was the principal aim of our study described in this MiniReview.

Before conducting genotoxicity tests, we selected a suitable cell line and optimized experimental conditions. BEAS-2B was the cell line of choice due to its non-tumor features and immortalization that allows almost non-restricted growth. To keep the cell line stable and to avoid the increased presence of morphologically aberrant cells (Fig. 21) with profound phenotypic impact [Zhao and Klimecki, 2014], we strictly adhered to recommended cultivation protocols. We also compared the stability of the % ABB in the control samples. We pooled the data from 54 controls and obtained the results with a very low standard deviation of the % ABB, indicating the high stability of the cells. Previously, we did these tests for A549 cells (human epithelial lung carcinoma; immortalized, but tumor cell line), but the level of DNA damage in the control sample varied in a broad range from experiment to experiment (data not shown). This comparison supports the limitation of the A549 cell line cited in the OECD guidelines [OECD, 2014]. Another important finding of our optimization steps is the concentration of cytochalasin-B. We found the optimal concentration to be only 1 µg/ml. This is lower than the published data for this cell line suggests; e.g. according to a recent review, the concentrations of cytochalasin-B required to induce cytokinesis should be in a range of 3–9 µg/ml. For these studies, however, no information on cytochalasin-B cytotoxicity is available [Gonzalez et al., 2011]. Cytotoxic effect was the reason to lower the cytochalasin-B concentration in our study.

The results of our experiments are generally in concordance with overall knowledge about the genotoxicity of diesel emissions and their major PAHs components. Moreover, in our study we observed comparable genotoxicity of extracts from diesel and biodiesel emissions, which supports the results of recent studies [Madden, 2015; Madden, 2016]. The similarity of the effects of diesel and biodiesel fuel extracts is also supported by a comparative analysis of the toxic responses of organic extracts from diesel and selected alternative fuels engine emissions in human lung BEAS-2B cells analysed using the whole genome expression approach [Libalova et al., 2016].

Recommendation for future research

Even though there is a relatively wide spectrum of human biomonitoring studies, focused on the effects of exposure to traffic on various population groups [DeMarini, 2013], *in vitro* studies analyzing the effect of exposure to engine exhaust on DNA damage in cell lines has not been systematically conducted, even though they have potential to be used for a broad spectrum of new motor fuels and new engine and exhaust after treatment technologies under various operating conditions. An urgent need for a comprehensive investigation of the impact on human health was

recently suggested for biodiesel exhaust due to a broad variety of the blends with different ratios of diesel and biodiesel components [Larcombe et al., 2015]. Direct injection gasoline engines (DISI or GDI), reported to produce relatively large amounts of ultrafine particles, along with a variety of alternative and emerging fuels (such as natural gas, LPG, ethanol and other alcohols), represent another urgent need. Studies related to the *in vitro* micronucleus test are almost completely missing, and the cell system presented in this MiniReview can serve as a suggestion for a broader application of the MN test in future studies.

Although the use of cell lines is a basic approach in genotoxic research, researchers need to be very careful in selecting optimal cell lines for genotoxicity tests. A cell line with a normal karyotype should be preferentially used, particularly due to the fact that the high diversity of chromosome number in individual cells of cancer cell lines can cause a variety between passages [Watson et al., 2004] and also impact analysis results, especially gene expression data [Dalerba et al., 2011]. Due to these facts, cancer cell lines cannot provide a meaningful interpretation of genotoxicity tests in relation to human health.

Analysis of MN, a frequently used method in genetic toxicology in both *in vitro* and *in vivo/ex vivo* studies, is generally preferably used for assessing the total micronuclei frequency without detailed information on MN content and origin. The identification of the type of MN as centromere positive or negative referring to the aneugenic or clastogenic effects of exposure to engine emissions is an important field for future research. Up to now, the advantages of recognition of MN type were utilized mainly in radiobiology, including biological dosimetry, for studying the association with some specific diseases, and only rarely for testing in genetic toxicology. The idea, concerning the evaluation of the type of MN by analyzing centromeric signals, was recently suggested in connection with evaluating the effect of PAHs in human lymphocytes, but can also be implemented in a wide field of biomonitoring studies, including *in vitro* studies using cell lines, where this type of research is completely missing [Sram et al., 2016].

Another challenge for accelerating the methodological approaches, which is connected with the very laborious visual scoring of MN, is the automation of this process. Significant achievements were recently reached in the field of automated image analysis of MN mainly in human lymphocytes [Fenech et al., 2013]. A semi-automated, fluorescent-based scoring protocol was also suggested for MN testing in human cells, even for adherent cell lines [Seager et al., 2014]. This application is unquestionably a challenge for accelerating future research, but both the advantages and disadvantages should be taken into consideration when selecting the optimal methodological plan for each cell line. The approach used in our above-mentioned study helped us to decrease the consumption of cultivation media and exclude cell harvesting,

but required visual scoring of MN. An alternative would be to use semi-automated MN scoring that would save time during MN analysis but for sample preparation higher media volumes would be needed, cells would have to be harvested, slides with appropriate cell density prepared and the classifier for the scoring optimized.

Last but not least, increasing the complexity of the studies and involving more methodological approaches in studies of the genotoxicity of engine emissions is desirable for future research. The methods required for understanding the changes in the mechanism of action and analyses of -omics biomarkers include transcriptomics, proteomics, metabolomics as well as investigating epigenetic markers (analysis of DNA methylation or microRNA expression).

6. Current and envisioned impacts of the project

Project results and project staff have contributed to the formation of the current plans to introduce monitoring of real driving emissions of nitrogen oxides and particulate matter in the EU.

Portable on-board emissions monitoring systems (PEMS) for vehicle emissions measurements were used to uncover excess emissions during real-world operation, including the first “DieselGate” involving heavy vehicles and excess NO_x emissions in the United States two decades ago, and including the “current” DieselGate affair with diesel automobiles.

Demonstration of a miniature PEMS serves as an enabling tool to extent real driving emissions measurements, established in the EU legislation for heavy-duty vehicles and planned for automobiles, to motorcycles and other smaller vehicles within the EU legislation.

Demonstration of online, on-road measurement of ammonia, nitrous oxide, and other pollutants of interest with a portable FTIR serves as an enabling tool for real driving emissions limits of such compounds within the EU legislation.

Demonstration of low-cost miniature particle sensor serves as an enabling tool for replacement or supplementation of the current opacity measurements during periodic vehicle inspections with another method capable of assessing the functionality of a diesel particle filter.

Project findings and background information gathered during the project have been used in citizen actions against unwise land-use choices that would have likely resulted in deterioration of air quality, including a construction of a large shopping center which would have brought large amounts of traffic into a historical city center.

Project findings on the reality of motor vehicle emissions have been implemented in many public lectures, including lectures at many conferences, but also at the Committee for Sustainable Transportation of the Czech Government, at political meetings, and at meetings and seminars for automobile repair technicians.

Toxicity assays demonstrated within the project have, along with different types of laboratory toxicity assays carried by several other groups, contributed to the increase in cooperation between engineering and toxicology groups in the field of a more direct assessment of effects of new fuels and technologies on health risks.

Ambient air measurements have sparked an interest from local authorities and citizen groups to assess air quality on a local (microscale) basis.

Technologies and methods demonstrated within the project are directly applicable to small home heating appliances, another distributed source of air pollution responsible for a large fraction of total particulate matter in the air.

New approaches developed within the project are now prepared to be applied for realistic assessment of engine emissions and their adverse health effects:

- Miniature and low-cost portable on-board monitoring systems is an enabling technology for extension of the monitoring of real driving emissions to small engines and to small or specialized non-road mobile machinery, where “classic” portable on-board monitoring systems are too bulky.
- Mini particle length detector offers an inexpensive way to detect malfunctions of diesel particle filters.
- Portable FTIR is an enabling technology for extension of the monitoring of real driving emissions to specific non-regulated pollutants of interest, arising out of new fuels and technologies.
- Mobile, time-resolved measurements of particle size distributions and particle number concentrations in ambient air allow for a more realistic assessment of particle-related health hazard than particle mass measurements used to date.
- Acellular tests of DNA adducts and oxidative damage may be used in future for high throughput analyses of toxic effects induced by engine emissions.
- Application and extension of the methods used for heavy diesel vehicles to gasoline engines, to small and non-road engines, and to home heating appliances has been planned in detail and is subject of several currently proposed projects.

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