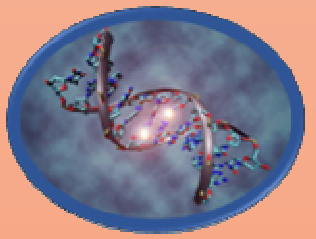
Characterization of complex nanoaerosol emissions
Health and Environmental issues

Biological tests for evaluation of toxicity of not only engine emissions

MEDETOX

Innovative methods of monitoring of diesel engine exhaust toxicity in real urban traffic
(LIFE10-ENV-CZ-651)
Project duration 1. 9. 2011 - 31. 8. 2016



Workshop – Rouen; March 24-25, 2015

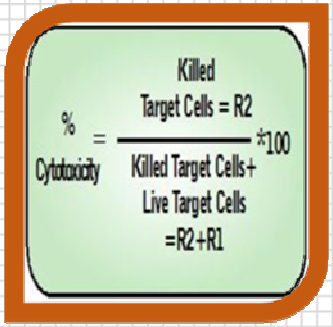
Andrea Rossnerova
Institute of Experimental Medicine AS CR, Prague, Czech Republic

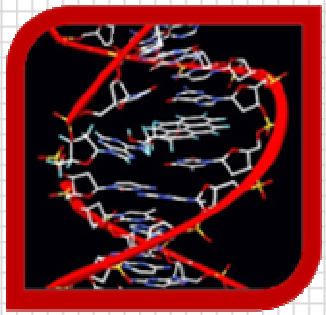
Outline of presentation

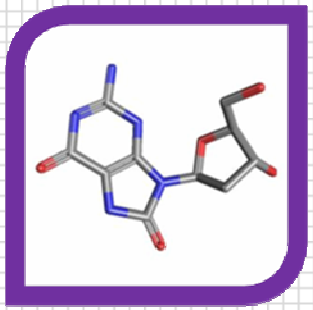
1. Nanoaerosol emissions and health effect – background

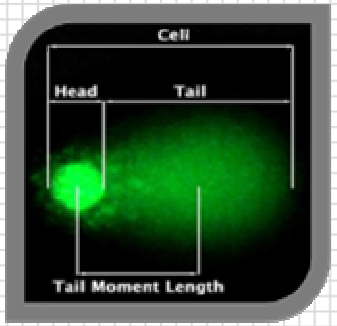
2. In vitro and in vivo systems for biological testing

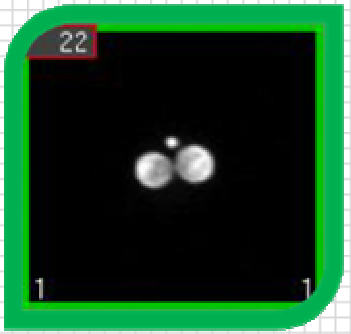
3. Biological tests (background, laboratory, results): cytotoxicity, DNA adducts, oxidative DNA damage, comet assay, micronucleus test, (-omics biomarkers)











Nanoaerosol emissions and health effects – background I

Nanoaerosol emissions are a part of air pollution produced by stationary sources (local heating and industry) and mobile sources (cars, trucks, buses, motorbikes, aircraft, boats, locomotives, farm equipment, lawn movers..)

Particulate matter (PM)

Coarse particles - < 10 μm

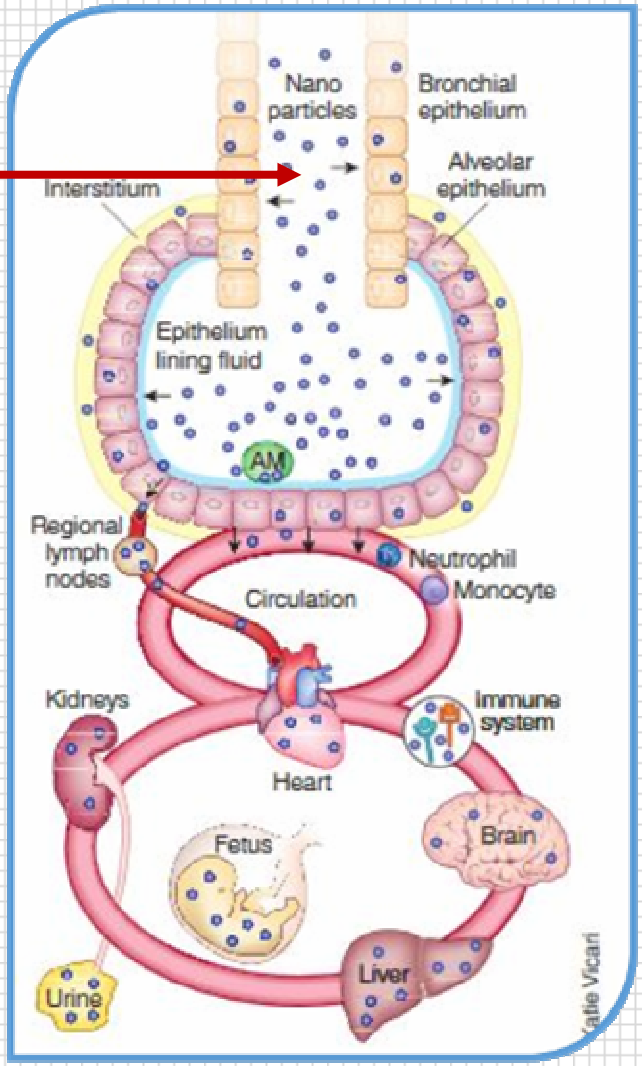
- deposited in the thoracic region of the lungs

Fine particles - < 2.5 μm

- penetrate the lung alveoli and cause inflammation

Ultrafine (Nano-) particles - <0.1 μm

- can enter various cells (tissues) via bloodstream as well as chemicals released from other PM and cause direct damage to macromolecules



Nanoaerosol emissions and health effect – background II

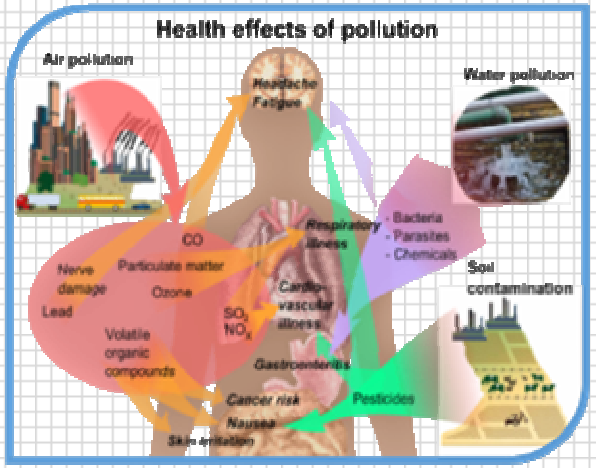
Increasing concentrations of air pollutants contribute to enormous amounts of adverse health outcomes worldwide.

Proof 1: 4 000 – 12 000 deaths estimated to be caused due to severe air pollution episode in 1952 in London (S. Henschel *et al.*, 2012).

Proof 2: Three times higher hospital admission for children after steel mill reopening in Utah Valley (S. Henschel *et al.*, 2012).

Proof 3: Exposure to ambient fine particles was recently estimated to have contributed to 3.2 million premature deaths worldwide in 2010, largely due to cardiovascular disease, and 223 000 deaths from lung cancer (IARC, 2013).
(3.7 million deaths in 2012 (WHO, 2014))

Some populations are more sensitive than others: children, elderly and pregnant mothers (prenatal exposure)

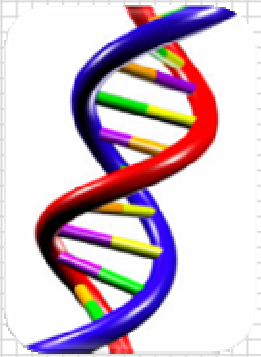


In vitro and in vivo systems

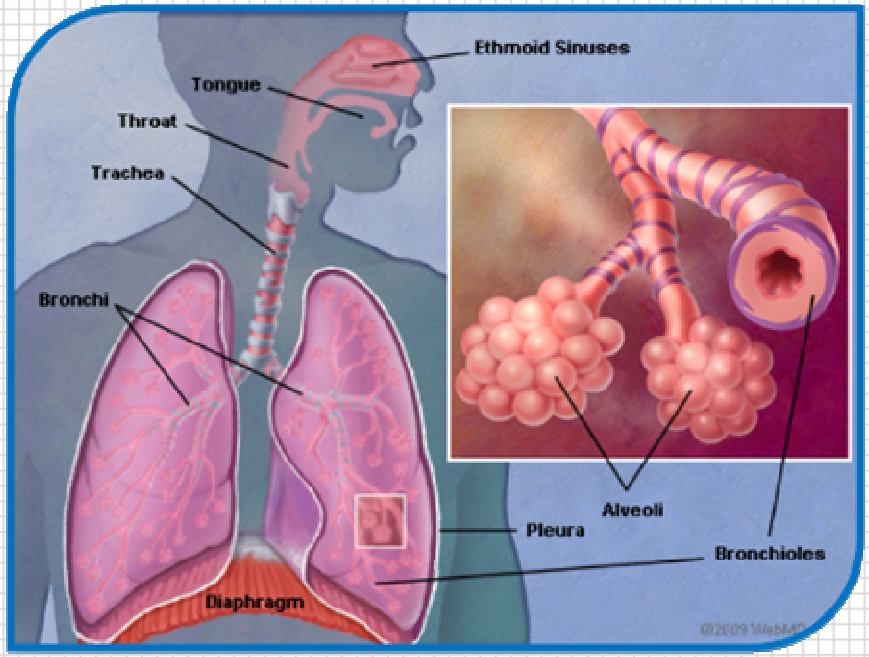
In vitro

Acellular tests

CT-DNA
Calf thymus DNA



Cellular tests



Cell lines from lung

HEL12469
Human embryonic fibroblasts cells

A549 cells
Human alveolar basal epithelial cells

BEAS-2B
Human bronchial epithelial cells

In vivo (ex vivo)

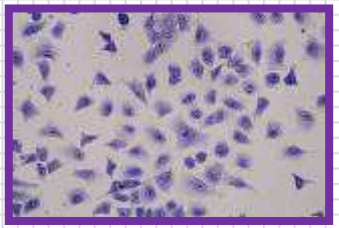
Blood
City policemen,
Bus drivers...

In vitro and in vivo systems - characteristics

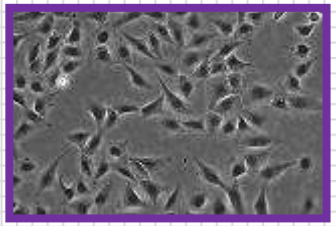
CT-DNA – calf thymus DNA: ds DNA isolated from thymus of calves, for acellular in vitro tests (DNA adducts and oxidative DNA damage), ideal system for testing of genotoxicity chemicals and mixtures like (B[a]P), EOMs from particulate matter collected from ambient air and/or EOMs from engine emission

HEL 12469 - normal human embryonic lung fibroblasts cells: non-tumor adherent cell line with normal karyotype for cellular in vitro tests (tested for all 5 methods), limitation in number of dividing during cultivation, low publication coverage in PubMed database

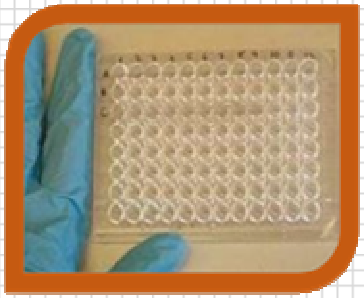
A549 - human alveolar adenocarcinoma cell line: adenocarcinoma hypotriploid cell line with modal chromosome number of 66, used for cellular in vitro tests (all 5 methods), interpretation is partly limited, adherent cells with unlimited dividing during cultivation, high popularity and publication coverage



BEAS-2B - human bronchial epithelial cells: originally non-tumor adherent cell line infected with an adenovirus 12-SV40 - ~75% cells with normal karyotype, long time cultivation in case of low cell density, used for cellular in vitro tests (currently tested), medium publication coverage



Blood – peripheral blood lymphocytes: normal cells for both in vitro (limitation due to differences between donors – variability through experiments) and in vivo testing through the population (lot of methods including – omics), high publication coverage



Biological tests – 1/1

$$\%\ \text{Cytotoxicity} = \frac{\text{Killed Target Cells} = R2}{\text{Killed Target Cells} + \text{Live Target Cells} = R2+R1} * 100$$

Cytotoxicity test(s) – background, laboratory

Definition: Cytotoxicity is the quality of being toxic to cells
Basic test in genetic toxicology, pharmaceutical industry...
Calculation of live and dead cells

Membrane integrity tests
-Trypane blue
-Propidium iodide
-Lactate dehydrogenase (LDH)

MTS assay

SRB assay

Clonogenic assay

Assays

ATP-based assay

WST assay

Determination of cytotoxic activity: WST-1 (stable tetrazolium salt) is used to determine the number of viable cells. Principle: bioreduction WST-1 to formazan (dark red) based on glycolytic production of NAD(P)H in viable cells. (ELISA reader – absorbance)

Biological tests – 1/2

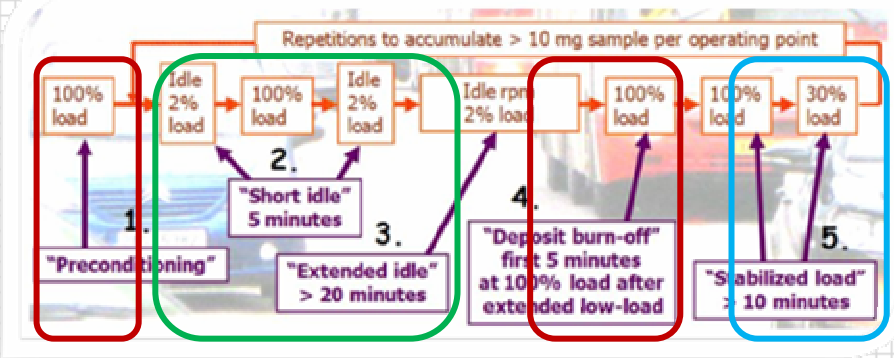
$$\%\ \text{Cytotoxicity} = \frac{\text{Killed Target Cells} = R2}{\text{Killed Target Cells} + \text{Live Target Cells} = R2 + R1} * 100$$

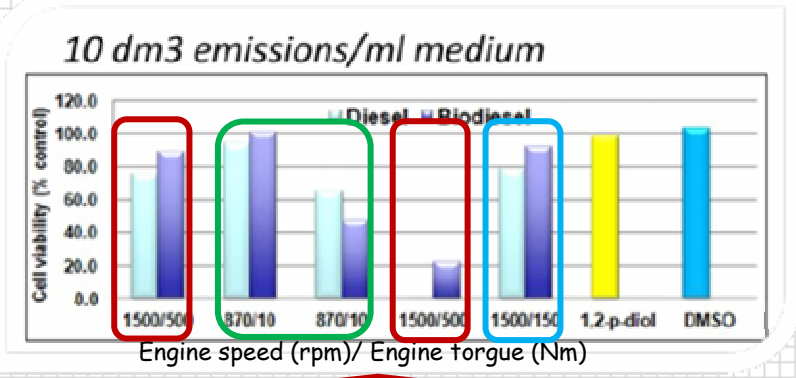
Cytotoxicity test(s) – results

TOXICITY OF DIESEL EMISSIONS UNDER SEVERE CONGESTION SIMULATED IN LABORATORY

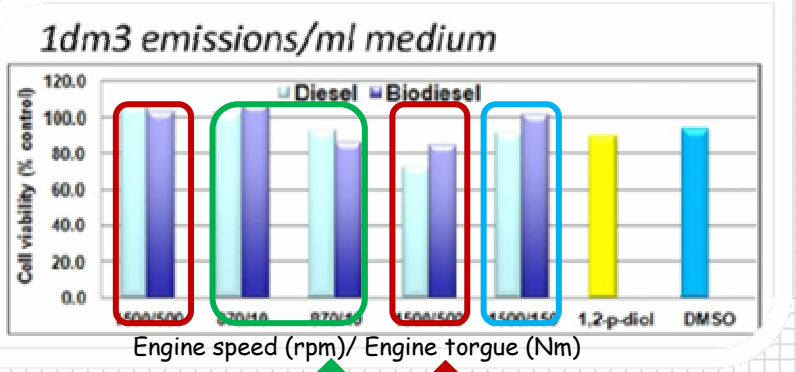
-Pilot study for verification of ability of biological tests in acellular (CT-DNA) and cellular system (A549)
-Engine – diesel Zetor 1505
-Fuels – diesel (EN 590) and 100% biodiesel (FAME)
-Emission from different phases of engine operation

A. ~ low-speed (short or ext. idle) – 2% load
B. ~ highway cruise – 30% load
C. ~ hill climb /acceleration – 100%load

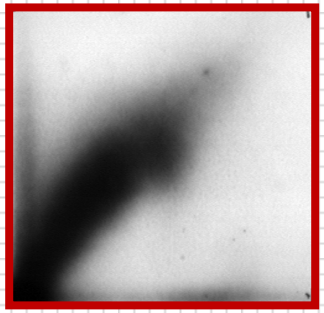




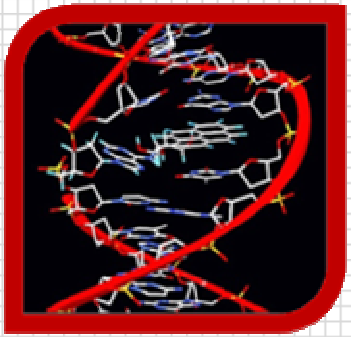
Cellular system - A549: Significant cytotoxicity was observed for higher dose of engine emissions



Slightly diminished viability after 24h exposure



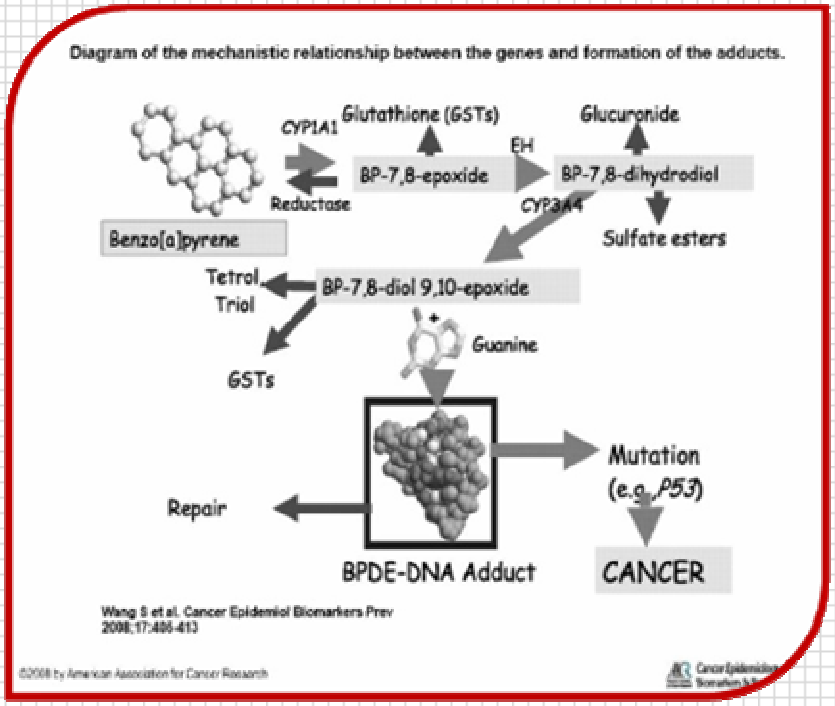
Biological tests – 2/1



DNA adducts – background

Method used from year 1975, more than 13 000 publications

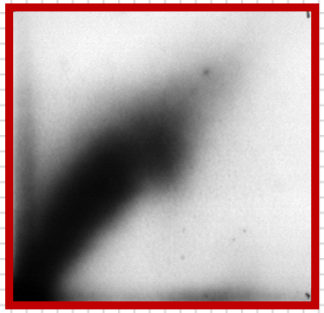
DNA adduct formation is one of the most frequent genotoxic events - covalent binding of the chemicals or its metabolites (BPDE) on nucleotides in DNA



Methodological approaches:

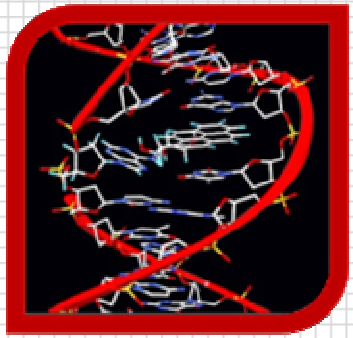
^{32}P-postlabelling
Mass spectrometry
Fluorescence methods
Immunoassays
Radiolabelled compounds (^{3}H, ^{14}C)

The methods differ by their sensitivity, specificity, applicability…
^{32}P-postlabelling is used in our laboratory in *in vitro* and *in vivo* studies



Biological tests – 2/2

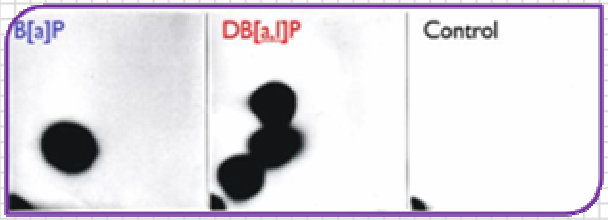
DNA adducts – laboratory

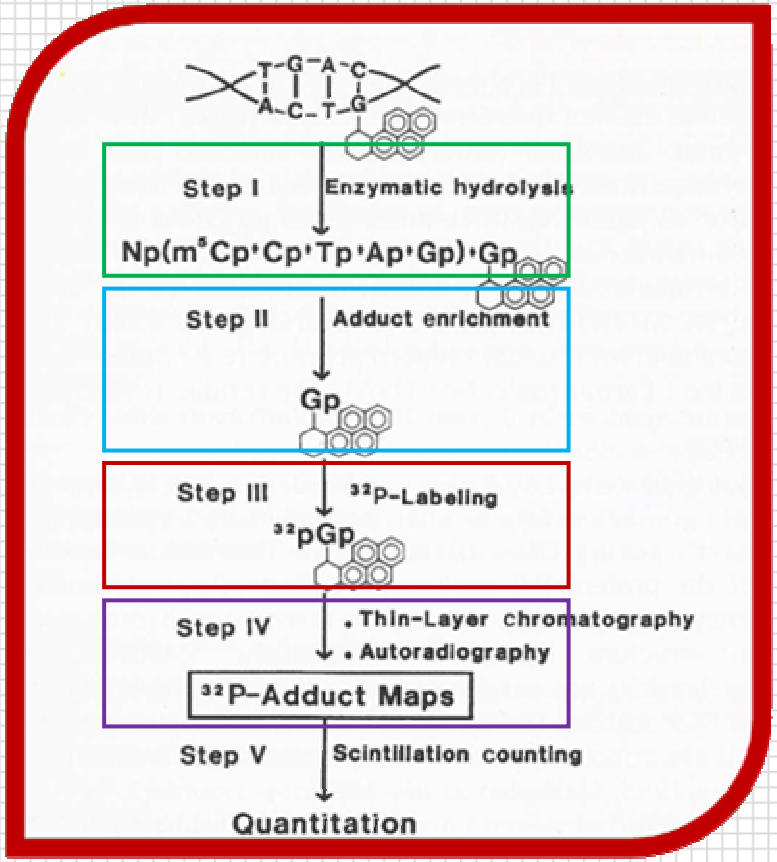


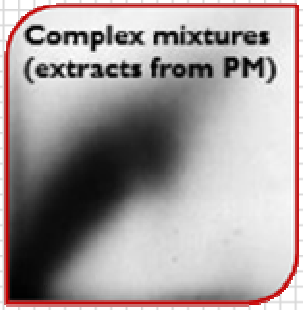
^{32}P-postlabelling method - very sensitive – 1 adduct per 10^9 normal nucleotides

Major methodological steps:

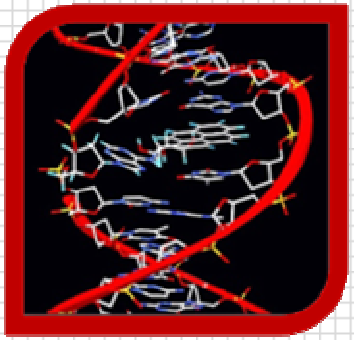
1.DNA isolation
2.Enzymatic hydrolysis
3.Adduct enrichment
4.^{32}P-labelling
5.Chromatography, Autoradiography
6.Quantitation – scintillation counting





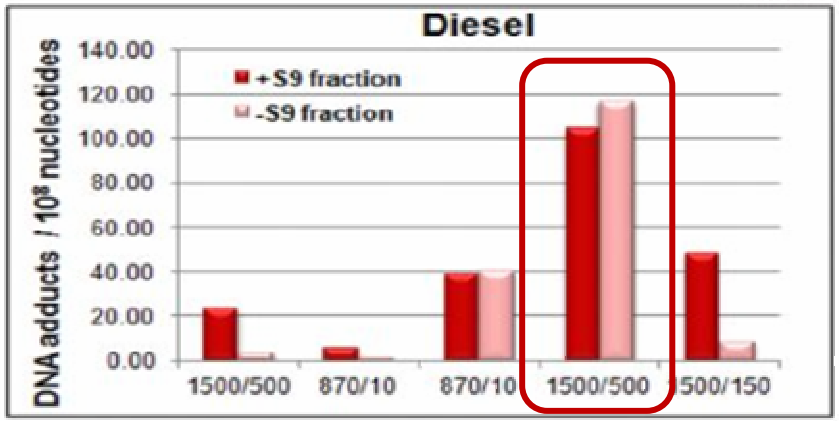


Biological tests – 2/3

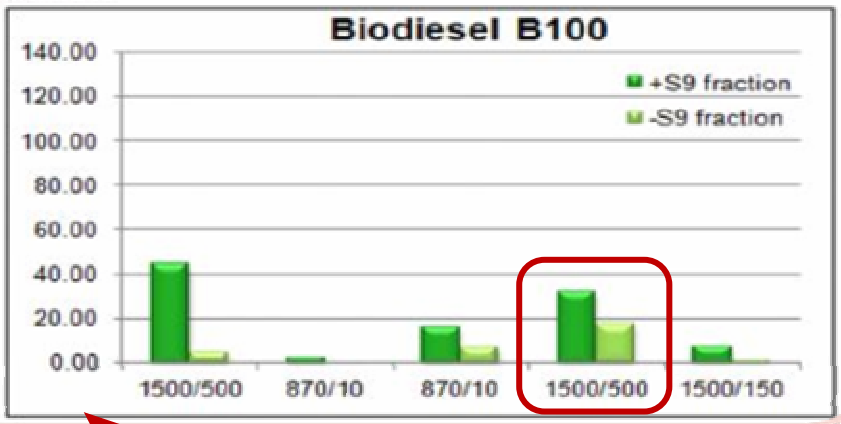


DNA adducts – results

ACELLULAR ASSAY: CT-DNA



DNA adducts



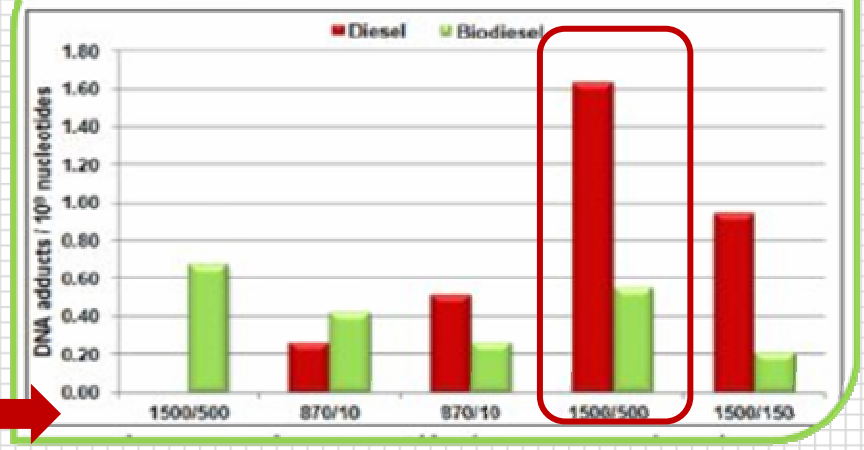
ACELLULAR ASSAY: CT-DNA
(10 dm³ emission/ml)

-Highest genotoxicity induced by operating mode 1500/500 (deposit burn-off), particularly for diesel.
-For biodiesel is genotoxicity substantional lower.

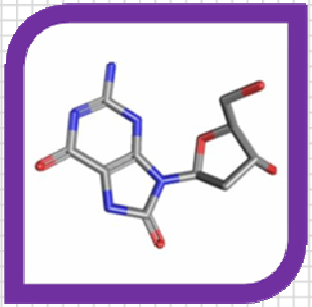
CELLULAR ASSAY: A549
(1 dm³ emission/ml culture medium)

- Similar to the acellular test, highest genotoxicity detected for engine operating mode 1500/500 (deposit burn-off) in combination with diesel.

DNA adducts CELLULAR ASSAY: A549



Biological tests - 3/1



Oxidative DNA damage- background (proteins and lipids)

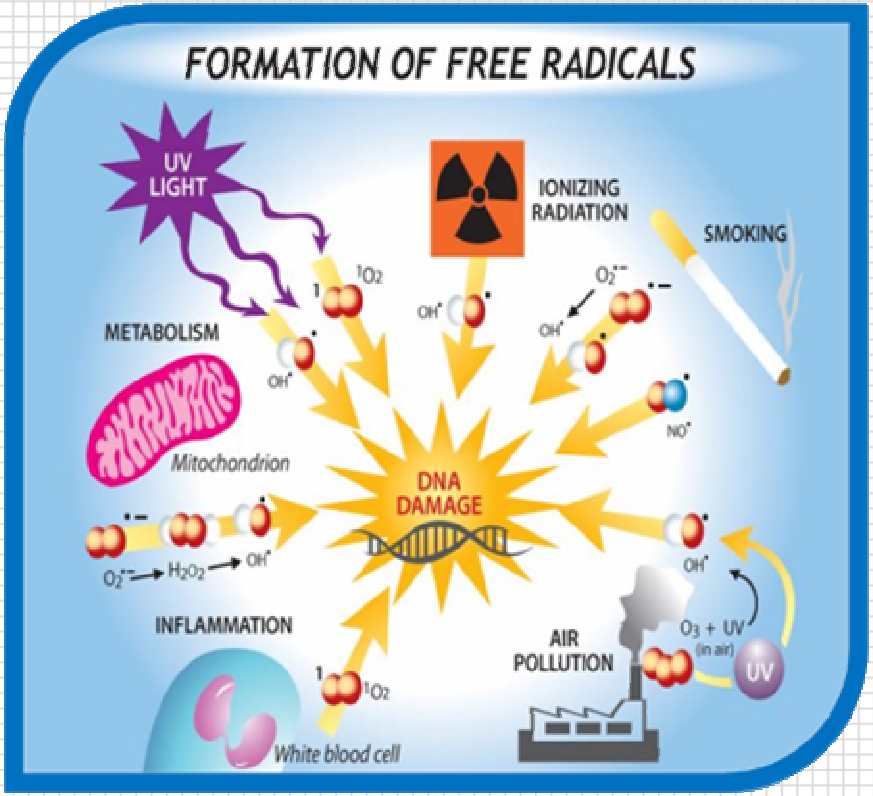
Oxidative stress - the result of an inbalance between levels of oxidants and antioxidants (widely studied - ~130 000 publications)

Reactive oxygen species ROS

radicals hydroxyl radical ($OH^{\cdot}$), superoxide ($O_2^{\cdot -}$), nitric oxide ($NO^{\cdot}$), lipid peroxyl ($LOO^{\cdot}$)

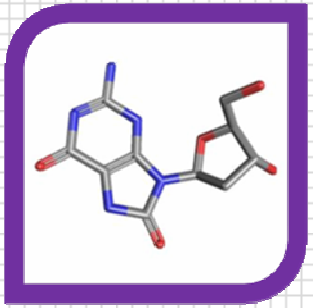
non-radical reactive oxygen derivatives peroxynitrite ($ONOO^-$), hypochlorous acid (HOCl), hydrogen peroxide (H_2O_2), ozone (O_3), and lipid peroxide (LOOH)

8-oxodG - the most abundant DNA lesion caused by ROS, highly mutagenic, causes in GC to TA transversions



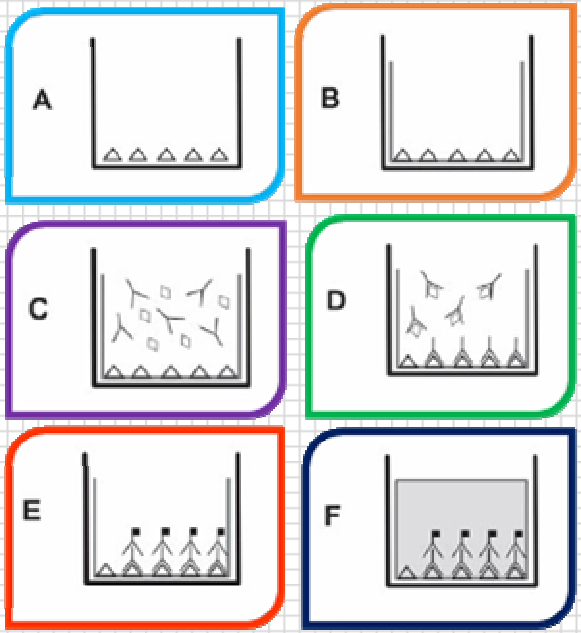
Biological tests - 3/2

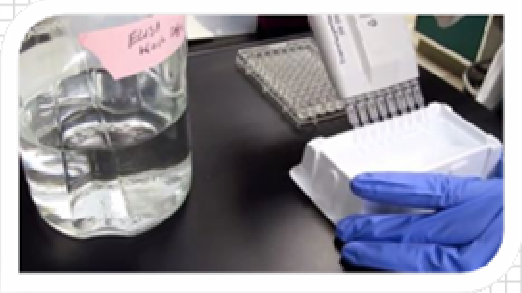
Oxidative DNA damage- laboratory



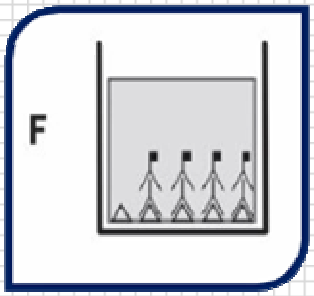
Methods for 8-oxodG detection:
HPLC-tandem mass spectroscopy
Competitive ELISA

Major methodological steps:
1.DNA isolation
2.A-**coating** with 8-oxoG-BSA
3.B-**blocking** with FCS
4.C-**incubation** with samples and primary anti-8-oxodG antibody
5.**D-competition**
6.E- **incubation** with secondary antibody conjugated with enzymes
7.F-**incubation** with chromogenic substrate and color development

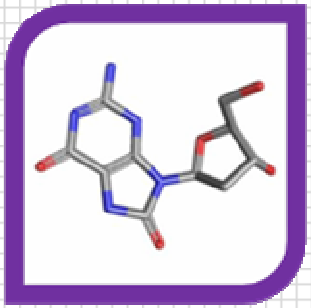




Rossner P. Jr., 2012

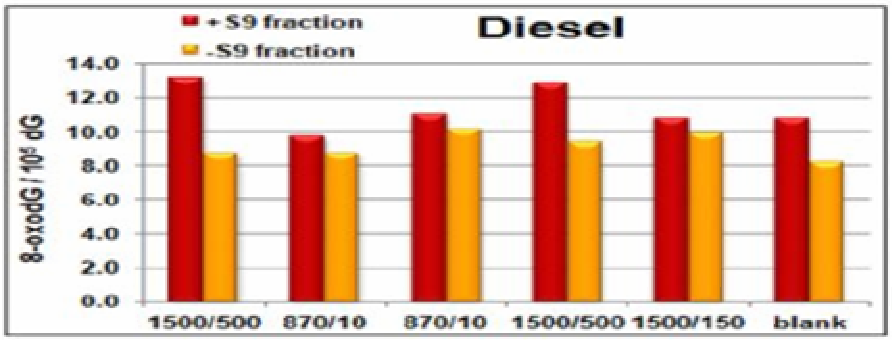


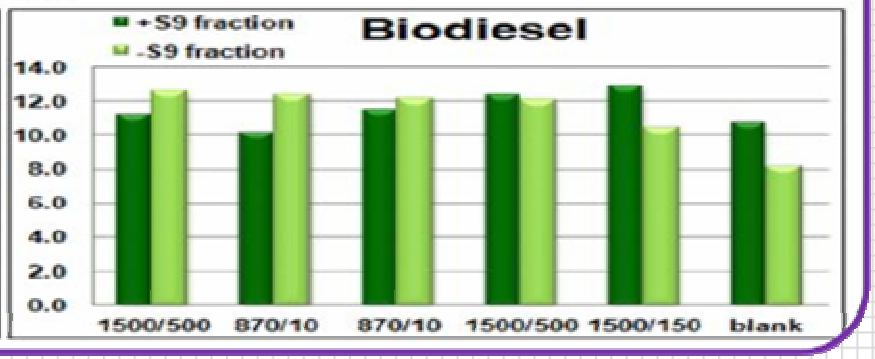
Biological tests - 3/3



Oxidative DNA damage- results

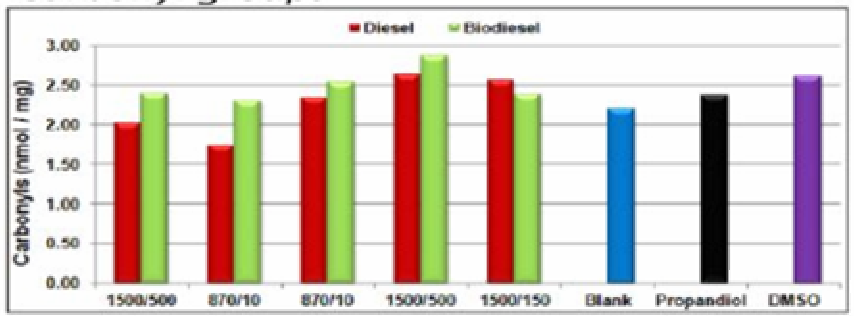
ACELLULAR ASSAY: CT-DNA 8-oxodG



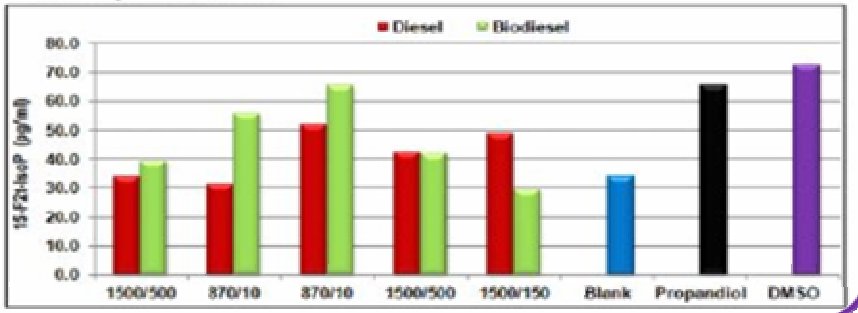


Oxidative damage of proteins and lipids- results

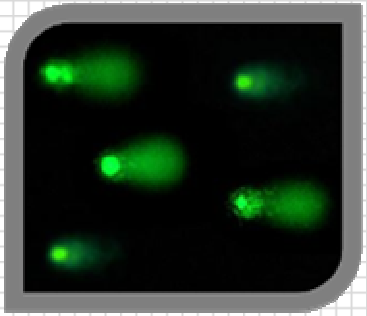
Carbonyl groups CELLULAR ASSAYS: A549



8-isoprostane

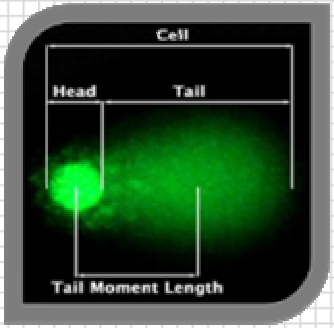


- No significant oxidative DNA damage (acellular assay) and damage of proteins and lipids (cellular assays) were observed for all operating modes for both diesel and biodiesel.
- **Repeatedly observed no significant induction of oxidative damage by organic extracts.**



Biological tests – 4/1

Comet assay- background



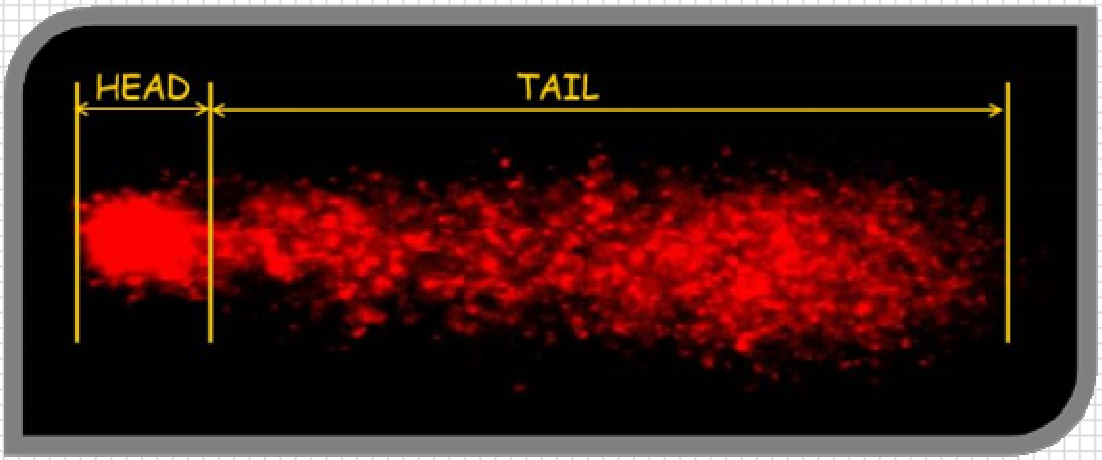
Method introduced between years 1984-1989, Single Cell Gel Electrophoresis (SCGE)

Simple and widely used method to study genotoxicity of chemicals including complex mixtures *in vitro* and *in vivo* (more than 8000 publications)

Assessment of DNA damage as a percentage of DNA in tail of comet from the total content of DNA in the nucleus

Alkaline version CA detects SSB or DSB

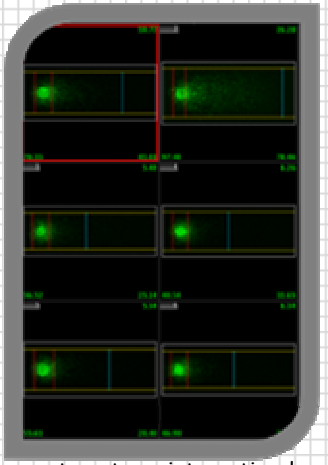
Breaks may also be introduced at the site of (oxidative) damage by treatment with enzymes such endonuclease (ENDO III) and glycosylase (FPG)



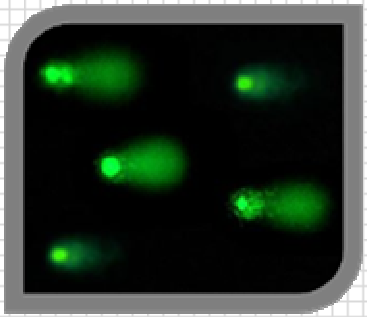
Various dyes for staining

50-100 comets

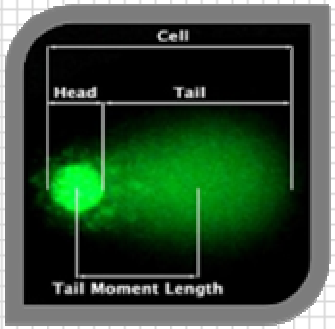
Possibility of automation



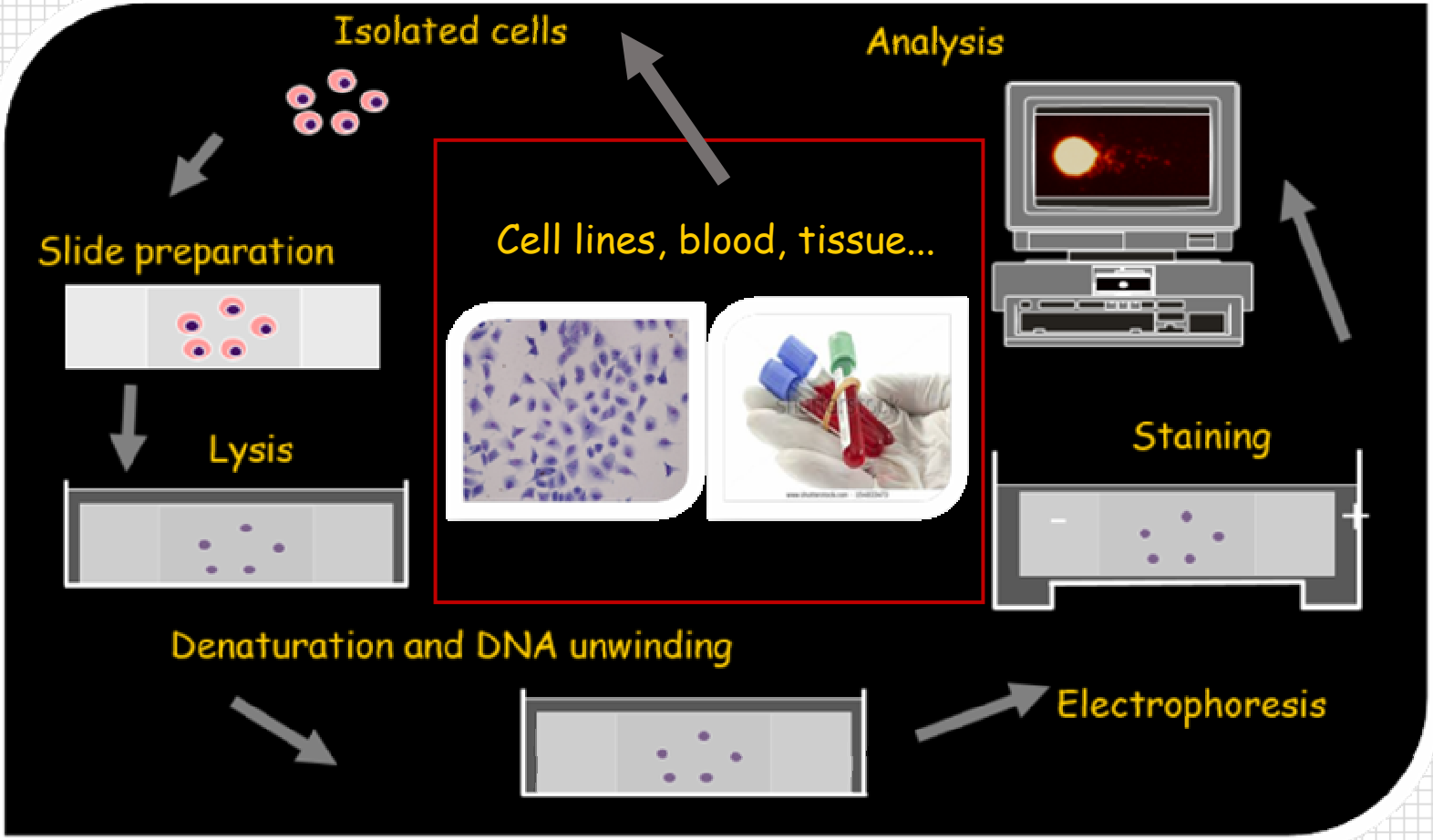
http://www.metasystems-international.com

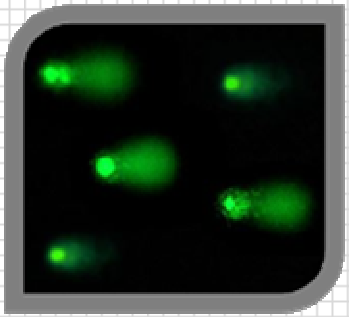


Biological tests - 4/2

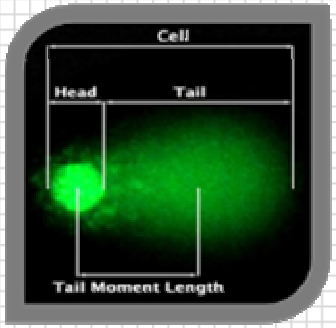


Comet assay- in laboratory





Biological tests – 4/3



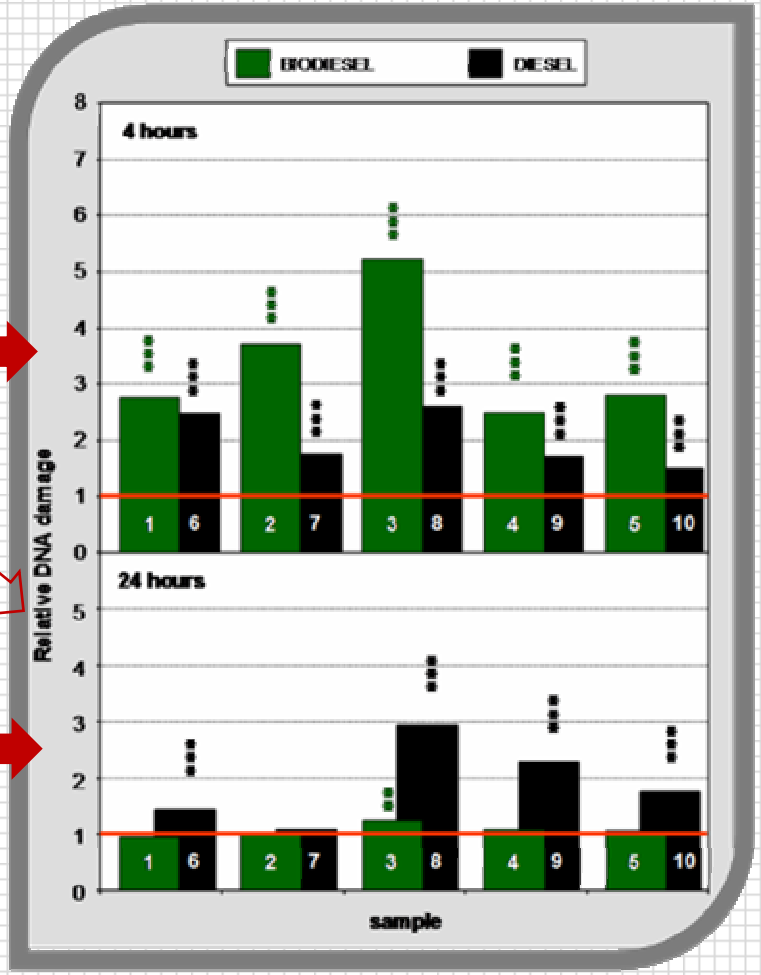
Comet assay- results

Total DNA damage in A549 cells exposed to **biodiesel** or **diesel** samples in relation to control values in cell culture treated only with solvent (DMSO).

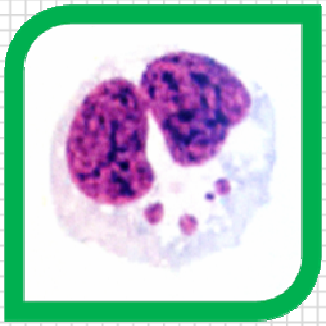
- 4 h exposure interval suggested a stronger genotoxic potential of **biodiesel** samples in comparison with diesel samples

- DNA damage was prevalently fully repaired during following twenty h.

higher level of DNA-SB of **diesel** samples at **24 h** than at 4 h interval.

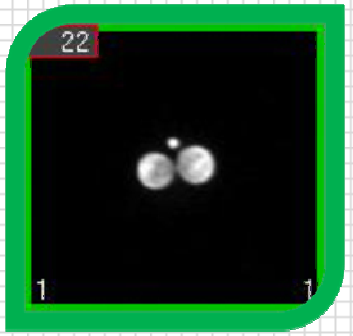


Red line indicates the control level = 1;
***p<0.001; **p<0.01; *p<0.05

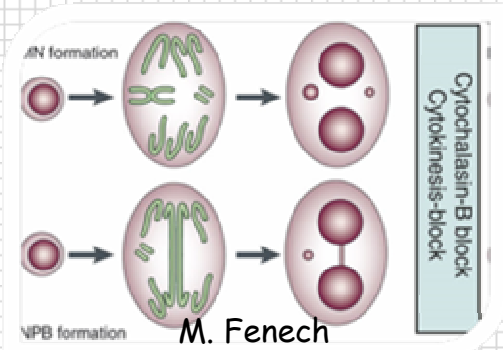


Biological tests - 5/1

Micronucleus test - background



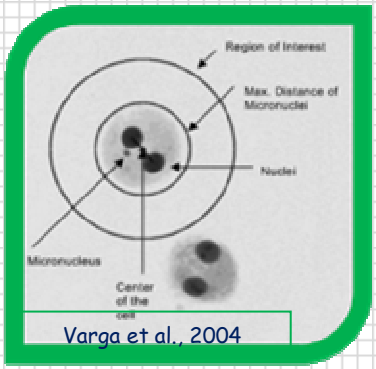
-method for evaluation of genotoxic effects of various chemicals, mixtures..... (chromosomal losses or breaks)



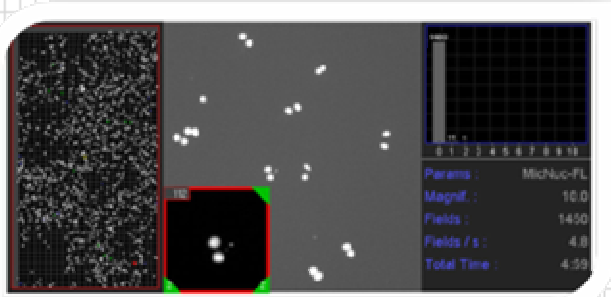
M. Fenech

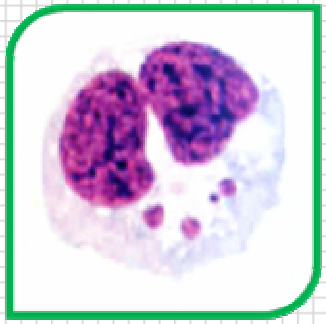
Visual analysis - from 1959 (Vicia faba), from 1976 - PBL, from 1985 - cytochalasin B (BNC), time-consuming method prevalenty Giemsa stained slides, cheep method

Automated analysis - from 1982 flow cytometry, from 1990 testing of image systems, from 2004 automated image analysis of DAPI stained slides - originaly for PBL (also for cell lines applications), allows the analysis of large numbers of BNC, scanning time ~ 5 min per slides

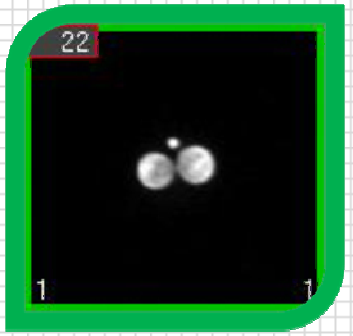


Varga et al., 2004



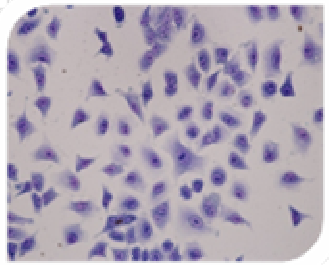


Biological tests – 5/2



Micronucleus test – in laboratory

Cell lines/blood cultivation, cytochalasin B adding



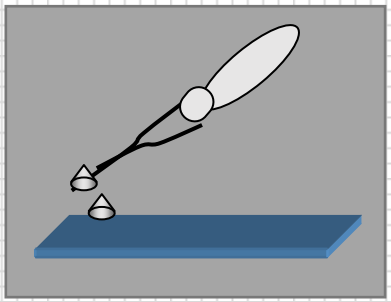
CYTOCHALASINE B

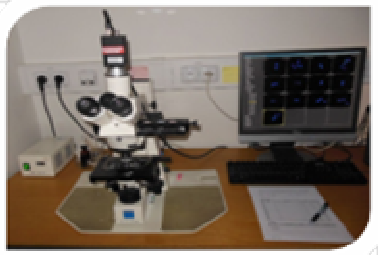
- a cell-permeable mycotoxin
-inhibits cytoplasmic division by blocking the formation of contractile microfilaments

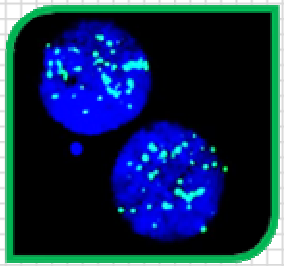
Harvesting and slide preparation, Giemsa or DAPI staining, microscopic analysis

Hypotonic solution

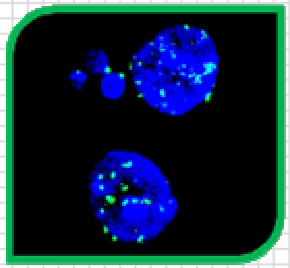
Fixation

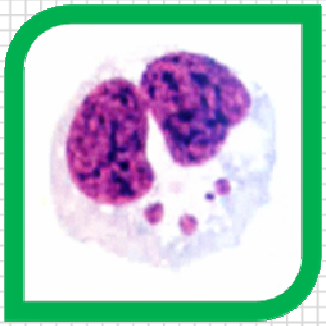




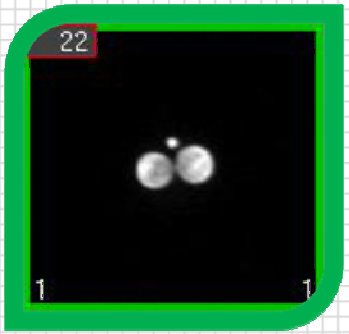


More information: Pan-Centromeric probes (aneugen x clastogen)
- bind to repetitive sequences that are specific to the centromeric regions for evaluation CEN+ and CEN- MN

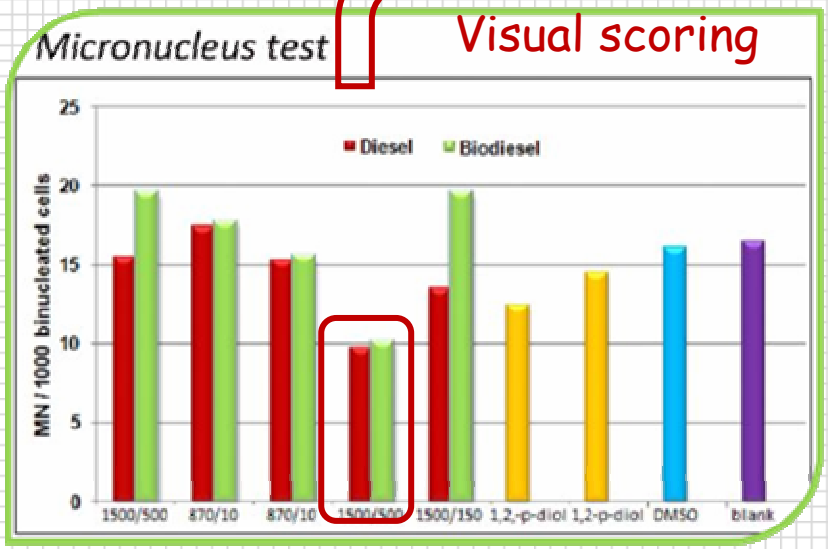




Biological tests – 5/3



Micronucleus test - results



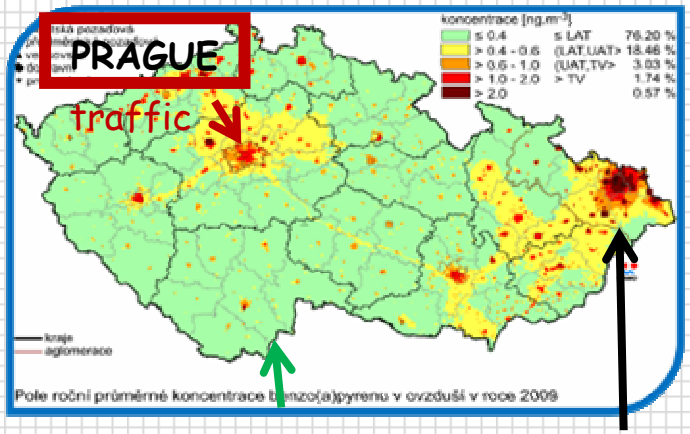
- „**Surprisingly**" – low frequency of MN in operating mode 1500/500 (deposit burn-off)
- Results were **confirmed by automated image analysis**
- **But** - decrease of CBPI to 1.69 in this operating mode in comparison with other samples where CBPI were detected almost equal to 2 (ideal), and lower frequency of detectable BNC
- lower viability as in cytotoxicity tests due to prolonged treatment with tested sample (4 h treatment with test compounds followed by 36 h of co-treatment of test compounds and cytochalasin B) in comparison with other tests

Method – timing: L. Gonzalez, B.J.S. Sanderson, M. Kirsch-Volders, **Adaptations of the in vitro MN assay for the genotoxicity assessment of nanomaterials**, Mutagenesis 26 (2011) 185-191.

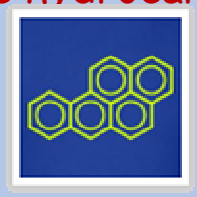
Common untypical cells: A549

Biological tests and -omics biomarkers in human population studies

Average year concentrations
of benzo[a]pyrene (B[a]P) in the air in 2009



Carcinogenic polycyclic aromatic hydrocarbons (c-



example: B[a]P (IARC: Group 1)

Exposure

Traditional biomarkers

1. Exposure
   (DNA adducts)
2. Effect
   (CCA, FISH, MN)
3. Susceptibility
   (SNPs)

-omics biomarkers of effect

1. Transcriptomics
   (mRNA)
2. Epigenomics
   (DNA methylation)

Micromics
(miRNA)

Disease

No differences in "Traditional biomarkers" (bulky DNA adducts, chromosomal aberrations, oxidative stress markers) between groups from Prague and Ostrava region

Involving -**omics** biomarkers

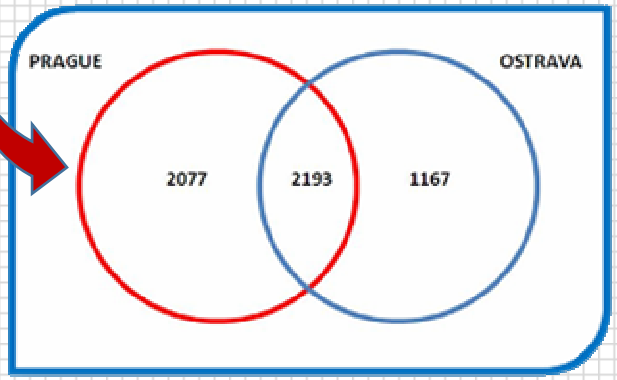
Biological tests and –omics biomarkers in human population studies

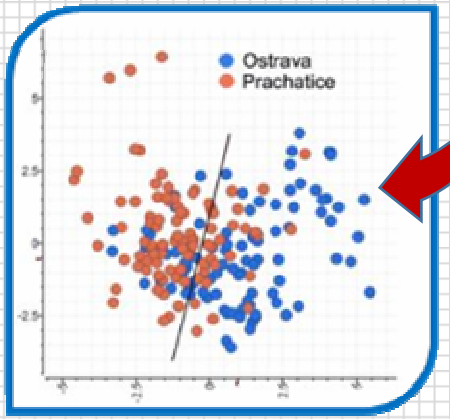
Global gene expression –GE (adults)
~ 48 000 transcripts

1. Leukocyte separation
2. RNA extraction and quality control
3. cDNA synthesis and IVT and labeling
4. Hybridization to chips and scanning

DNA methylation (children)
~ 27 000 CpG

Methylation of cytosine in CpG sites of DNA is linked to control of gene functions
↑ methylation in promoter = ↓ GE
DNA conversion with sodium bisulfite+array





Both – gene expression and DNA methylation differed between locations

This result suggests an adaptation of human population to high levels of air pollution

Differencies in regulation including miRNA analysis is task for future research

Conclusions

All described biological tests: cytotoxicity, DNA adducts, oxidative DNA damage, comet assay, micronucleus test belongs to to the standard battery of tests in genetic toxicology

New experience from human biomonitoring studies (-omics biomarkers: GE, miRNA and epigenetics as a whole) can be also utilized in future *in vitro* studies focused e.g. on genotoxicity of engine emissions for evaluation of changes in concrete pathways.

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BIOTOX (13-01438S)

Thank you for your attention!