

Mutagenicity of diesel exhaust – what is the most reliable *in vitro* test?

Sandro Steiner¹, Jan Czerwinski², Pierre Comte², Andreas Mayer³, Norbert Heeb⁴, Alke Fink¹, Barbara Rothen-Rutishauser¹

¹Adolphe Merkle Institute, University of Fribourg, Switzerland. ²Bern University for Applied Sciences, Switzerland. ³TTM, Technik Thermischer Maschinen, Switzerland. ⁴EMPA, Swiss Federal Laboratories for Materials Testing and Research, Switzerland.

In June 2012, the International Agency for Research on Cancer (IARC) classified diesel engine exhaust as a group 1 carcinogen to humans. In the according press release, the IARC stated that with this classification 'governments and other decision-makers have a valuable evidence-base on which to consider environmental standards for diesel exhaust emissions and to continue to work with the engine and fuel manufacturers towards those goals'. It was also stated, however, that whilst successively stringent emission legislations may result in reduced emissions, it is not clear how this translates into health effect improvements and that research into this direction is needed.

Three possible approaches for this research exist: epidemiological, *in vivo* (human and or animals) and *in vitro* studies. Among these three, only *in vitro* studies are of sufficiently low cost, free of ethical concerns, allow for a

high degree of standardization and for high throughput screening of various exhausts produced from different engine types under different operation conditions.

A literature search on studies investigating the mutagenicity of diesel exhaust *in vitro* reveals that most studies were performed using the bacterial Ames-test (Ames et al. 1973, Maron and Ames 1983, Figure 1). The bacterial Ames-test uses genetically modified prokaryotic organisms to measure the genotoxic and hence mutagenic potential of a test substance. The principle relies on mutations that have been artificially introduced into the *his*-operon (the genetic sequence which encodes the proteins necessary for the biosynthesis of the amino acid histidine) and render

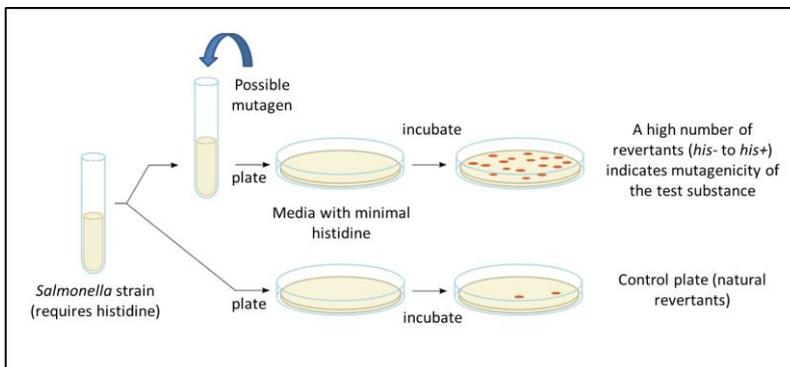


Figure 1: principle of the Ames-test. *his*⁻ bacteria are treated with a possible mutagen and incubated on minimal-histidine media, allowing for limited growth and incorporation of mutations into the genetic material for a short period of time. During the following incubation on histidine-free media, only revertants (*his*⁺ bacteria) are able to grow and form colonies. The number of revertants is compared to the number of natural revertants in an untreated control

the bacteria unable to grow on histidine-free medium (*his*⁻ bacteria). A test substance with high genotoxic/mutagenic potential will cause mutations in the bacterial genome, some of which will re-establish the functionality of the *his* operon *i.e.* result in the conversion of *his*⁻ to *his*⁺ bacteria. The output of the test is therefore the number of revertants in response to the treatment, that is, the number of bacterial colonies that, due to mutations in their genome, became able to grow on histidine-free medium.

However, because of different modes of DNA storage, -protection and -repair the Ames-test may fail to reflect mutagenicity in non-bacterial organisms, e.g. humans. Further, many studies conducted in this field so far used organic extracts from diesel exhaust particles, which bears a high risk of biased sample generation and neglects effects of gaseous exhaust components, non-extractable species and the solid carbonaceous or metal particle cores. Finally, even when using complete exhaust, the Ames-test may fail to yield results representative for human toxicology, since bacteria and human cells do not interact with particles in the same way (Clift et al. 2012). Most importantly, it has been shown that diesel exhaust particles are not taken up by the bacteria most commonly used in the Ames-test (*Salmonella typhimurium*), whereas uptake in human lung cells occurs. This implies that a subset of organic and/or inorganic chemical species adsorbed to the particle surface and the redox-active surfaces of the particles themselves may not be able to interact with the genetic material of bacteria whereas in eukaryotic cells they do. Last but not least, the simplicity of a bacterial test system is problematic, since it cannot account for mutagenic effects resulting from the mere presence of particles: the induction of pro-inflammatory responses with the concomitant endogenous production of DNA damaging reactive oxygen species (secondary genotoxicity, Schins and Knaapen 2007).

In ongoing research we are testing whether the Ames-test is a suitable method for exhaust mutagenicity testing and if yes, how it is best used for this purpose. In a first approach, we were interested in how the mutagenicity of organic extracts obtained from sampled exhaust particles compares to the mutagenicity of complete exhaust. In a well-established exhaust exposure system usually used for exhaust exposure experiments with human lung cell cultures (Müller et al. 2010, Steiner et al. 2012), we exposed bacterial cultures (*Salmonella typhimurium*, strain TA100, Xenometrics Ames MPF Mutagenicity assay (microplate format)) to ten-fold diluted complete diesel exhaust produced by an Opel Astra X20DTL at constant velocity of 35 km/h. Exhaust dosing was achieved by varying the exposure duration between 2, 4 and 6 hours. Two different types of exhaust were used: exhaust produced from standard low Sulfur diesel (LSD, Greenergy), and exhaust produced from the same diesel to which 40ppm Satacen[®], an iron based fuel additive (LSD + Satacen) had been added.

Simultaneously to the exhaust exposures, diesel exhaust particles were collected on PallFlex filters using a constant volume sampling system (CVS, Horiba PMU 9000). From the collected particles, organic extracts were produced by Soxhlet extraction using 100 ml dichloromethane as a solvent. The extraction was performed at 50°C for 22 hours with 40 minute cycles (33 cycles). The solvent was removed by rotary evaporation and the extractable organic fractions were redissolved in dimethylsulfoxide and stored at 4°C in the dark until further use. Based on the exhaust dilution used for complete exhaust exposure (1:10) and

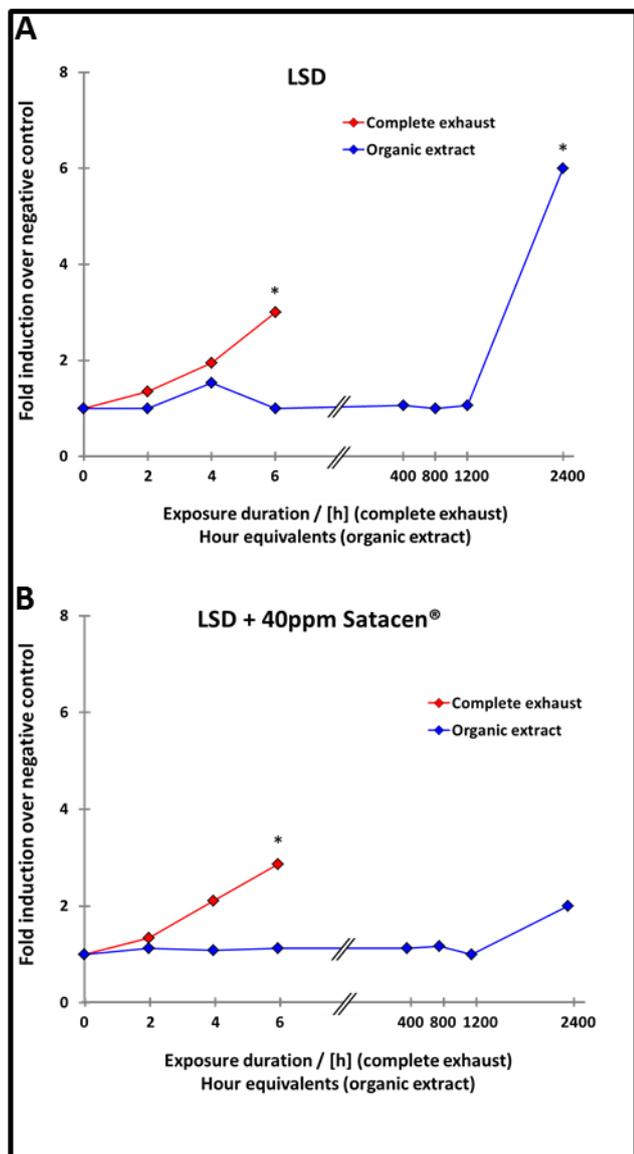


Figure 2: comparison of the mutagenic potential of complete exhaust and organic extracts obtained from collected exhaust particles. A) standard low sulfur diesel, B) low sulfur diesel containing 40 ppm Satacen[®]. For complete exhaust exposures, the x-axis indicates the exposure duration in hours, for the extract exposures it indicates the number of exposure-hour-equivalents.

extraction was performed at 50°C for 22 hours with 40 minute cycles (33 cycles). The solvent was removed by rotary evaporation and the extractable organic fractions were redissolved in dimethylsulfoxide and stored at 4°C in the dark until further use. Based on the exhaust dilution used for complete exhaust exposure (1:10) and

particle sampling (1:6), the exhaust volume-flow through the exposure chamber (2 l/min) and the CVS (40 l/min), the duration of exhaust exposure/particle sampling (2, 4, 6 hours and 10 minutes respectively), the total inner surface area of the exposure chamber (2900 cm²), the surface area of the bacterial cultures (2 cm²), the particle deposition rate in the exposure chamber (about 0.5% as determined in previous studies (Müller et al. 2010, Steiner et al. 2012) and the mass of particles collected on the PallFlex filters, exposure-hour equivalents of organic extracts were calculated: one exposure-hour-equivalent corresponds to the amount of organic extract that was derived from the amount of particles that deposited on the bacterial cultures during one hour of exposure to complete exhaust.

For both modes of Ames-test experiments, the bacterial cultures were incubated for 48 hours after the exposure and the number of revertants in the exposed samples was compared to the number of natural revertants (*i.e.* revertants in the untreated control).

The results are shown in Figure 2. Exposure to complete exhaust resulted in significant mutagenic effects after 6 hours for both, LSD and LSD + Satacen (a three-fold change in the number of revertants relative to the negative control can be considered significant), whereas exposure to organic extracts did so only when more than 1200 exposure-hour-equivalents were applied. We can therefore state that Ames-test results obtained from organic extracts vastly underestimate exhaust mutagenicity (more than 200-fold), which indicates that the gaseous exhaust components and/or the particulate fraction make an important contribution.

Satacen decreased the mutagenicity of the organic extracts, indicating that the mutagenicity of at least the extractable particle-adsorbed fraction of organic semi-volatiles was affected. The exhaust produced from LSD + Satacen contained a considerably higher number of particles (resulting from the additive itself) but, similarly as the organic extract, was found to be less mutagenic as LSD-exhaust. This suggests that the expected mutagenicity of the particulate exhaust fraction was not properly accounted for by the Ames-test.

We therefore conducted a first series of further exposure experiments with the same two fuel types, but with a silicon carbide diesel particle filter (DPF) installed at the tailpipe of the test vehicle (IBIDEN, 200 cpsi, pore size

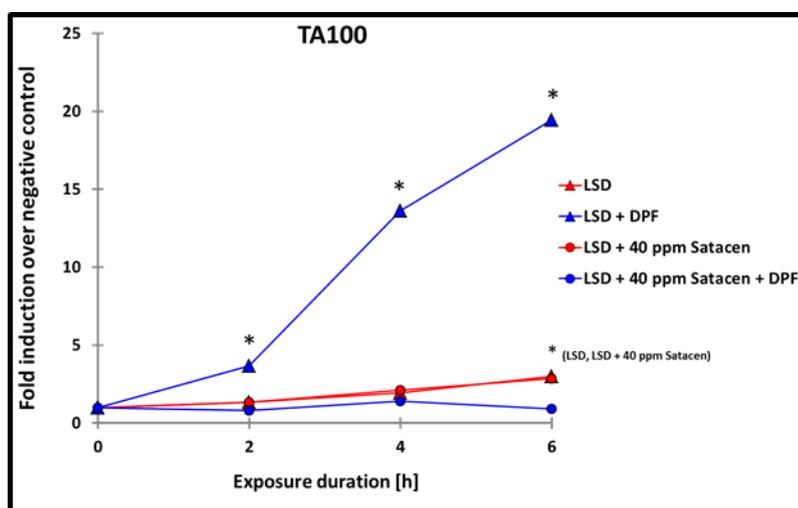


Figure 3: mutagenicity of exhaust produced from low sulfur diesel (LSD), LSD containing 40 ppm Satacen 3® (LSD + 40 ppm Satacen), and the same two exhaust types after filtration by a wall-flow diesel particle filter (LSD + DPF, LSD + Satacen + DPF)

10 µm with narrow pore-size distribution, obtained from a Peugeot 406, the oxidative catalyst had been removed). The results of these experiments are shown in Figure 3. We found that the DPF considerably increased the mutagenicity of LSD-exhaust, but decreased the mutagenicity of LSD + Satacen-exhaust.

We hypothesize that one reason for this result was the absence of agglomeration mode particles in the filtered exhaust, *i.e.* the absence of available surface for the condensation of semi-volatile organics, resulting in the formation of condensed, non-particle adsorbed organics downstream the DPF. These organic species can be assumed to be more available to bacteria, since as soon as they are dissolved in the culture medium, they are relatively free to pass through

the bacterial cell wall, which they would not be when adsorbed to solid particles. In contrast LSD + Satacen + DPF-exhaust acted less mutagenic than LSD or LSD + Satacen-exhaust. On one hand, this could indicate a particle-related mutagenic effect of the LSD + Satacen-exhaust, or a more efficient detoxification of the organic exhaust fraction by the additive on the filter on the other.

In summary, we could show that the Ames-test strongly underestimates exhaust mutagenicity when organic extracts of exhaust particles are used for exposure instead of complete exhaust. The question however,

whether and to what extent this effect relies on the absence of the particulate fraction in the extracts could not be answered so far.

In future experiments, we will compare Ames-test results on exhaust- and extract-mutagenicity to results obtained by expressional analysis of relevant genes involved in DNA-repair (Qiagen RT² Profiler PCR Array) in a more complex biological system, *i.e.* a triple-cell co-culture model of the human respiratory epithelium (Blank et al. 2007, Rothen-Rutishauser et al. 2005, 2008). This will give more insight into the suitability of the Ames-test and organic extracts for exhaust mutagenicity measurement with respect to human health. Further, since the triple cell co-culture model contains immune cells and is therefore capable of showing pro-inflammatory reactions, by repeating the experiments with filtered exhausts using these cell cultures, we will obtain more information on what contribution to the overall exhaust mutagenicity is made by the particle fraction via mechanisms of secondary genotoxicity.

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¹Adolphe Merkle Institute, University of Fribourg, ²Bern University of Applied Sciences, ³TTM Technik Thermische Maschinen, ⁴Swiss Federal Laboratories for Materials Testing and Research

Background

The bacterial Ames-test (1, 2) is among the most widely used methods for mutagenicity measurement of engine exhaust. Most commonly however, the mutagenicity of organic exhaust particle extracts is described, omitting effects of volatile and non-extractable components and exhaust particles. Furthermore, it is debated, whether the Ames-test can properly account for effects of particulate matter at all (3), and the extrapolation of results obtained in bacterial systems to humans is problematic

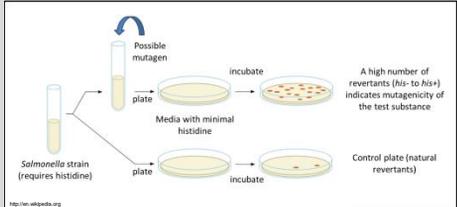
In ongoing work, we are trying to identify the most reliable *in vitro* approach for mutagenicity-testing of engine exhaust. Currently, we are testing to what extent the bacterial Ames-test is able to account for particle related effects and whether the mutagenicity of organic extracts is comparable to the mutagenicity of complete exhaust.

The bacterial Ames-test

Salmonella typhimurium carrying point mutations in the *his*-operon, unable to synthesize the amino acid histidine.

Mutagens may restore the function of the *his*-operon

-> revertants able to grow on histidine-free media



Pros and cons

- + Fast, robust, low-cost, only limited equipment and skills needed
- Very simplistic, extrapolation of results to humans is questionable, no uptake of exhaust particles in bacteria (Figure 1)

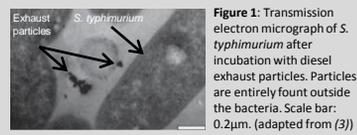
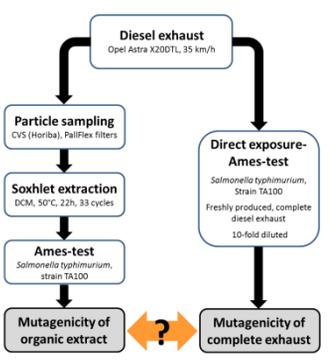


Figure 1: Transmission electron micrograph of *S. typhimurium* after incubation with diesel exhaust particles. Particles are entirely found outside the bacteria. Scale bar: 0.2µm. (adapted from (3))

Part 1

Can exhaust mutagenicity be described using organic particle extracts?

Experimental approach:



Two exhaust types are tested:

- Low sulfur diesel (LSD)
- Low sulfur diesel + 40 ppm Satacen® (iron based fuel additive, LSD + 40 ppm Satacen)

Exhaust/extract exposures:

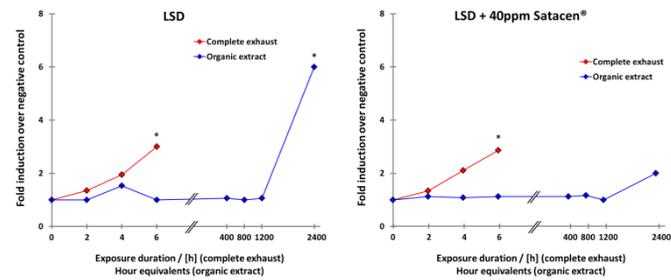
Complete exhaust:

- Dosing via exposure duration

Organic extracts:

- Dosing via particle mass, expressed in exposure-hour-equivalents*

Results:



- Positive results (*) are obtained already after 6 hours exposure to complete exhaust, but only after more than 1200 hour-equivalents of organic extracts
- The results are comparable for the two exhaust types. Satacen slightly reduces exhaust mutagenicity.

Conclusion:

Organic extracts underestimate diesel exhaust mutagenicity **more than 200-fold!**

* exposure-hour-equivalent: the amount of organic extract obtained from the amount of particles that deposit on the bacterial cultures during one hour of exposure to complete exhaust. Calculated based on the particle deposition rate in the exposure system during complete exhaust exposures, the exhaust volume used for complete exhaust exposure, the particle mass on the Pallflex filter used for extraction and the exhaust volume used for particle sampling.

Part 2

Can the Ames-test account for particle-effects?

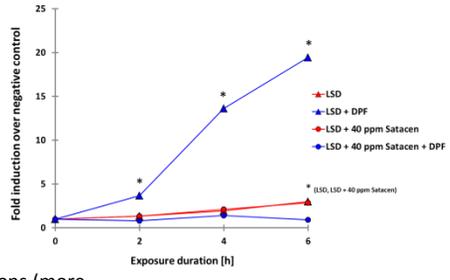
Experimental approach:

Ames-test (direct exposure) with unfiltered (LSD, LSD + 40 ppm Satacen) and filtered exhaust (LSD + DPF, LSD + 40ppm Satacen + DPF).

Results:

The DPF increases the mutagenicity of LSD-exhaust.

The increased mutagenicity may result from the absence of condensation surface in the filtered exhaust -> condensated non-particle-adsorbed hydrocarbons (more available to bacteria). The same would apply for LSD + 40 ppm Satacen + DPF, but the additive reduces the mutagenicity of the condensed organic exhaust fraction.



Hypothesis to be tested: In a system able to respond to particles (i.e. able for particle-uptake, such as human lung cell cultures), the DPF will not increase exhaust mutagenicity, or may even cause a decrease.

Future perspectives

Further comparison of the mutagenicity of complete exhaust and organic extracts using human lung cell cultures as biological test-system.

Testing how exhaust filtration affects mutagenicity towards human lung cell cultures

Investigating the contribution of secondary genotoxicity (brought by reactive oxygen formation due to pro-inflammatory reactions) to the overall mutagenicity of engine exhausts