# Cytotoxicity and genotoxicity properties of particulate matter fraction 2.5 µm

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**Abstract.** In the ambient is more than 2,000 chemical substances, some of them are absorbed on the surface of the particulate matter and may causes many health problems. Air pollution is responsible for more than 3.2 million premature deaths which classifies it as a second place environmental risk factor. Especially dangerous for health are polycyclic aromatic hydrocarbons and their nitro- and amino derivatives which shows mutagenic and carcinogenic properties. Air pollutions were also classified by International Agency for Research on Cancer to group which carcinogenic properties on human were proved by available knowledge. Air pollutions, including particulate matter are one of the biggest problem in Polish cities. World Health Organization in report published in May 2016 set many of Polish cities on the top of the list most polluted in European Union. The article presents results of mutagenicity, genotoxicity and cytotoxicity researches conducted on a particulate matter fraction 2.5 µm collected during all year long in Wroclaw agglomeration. The material were collected on filters using high-flow air aspirator and extracted using dichloromethane. Additionally it was fractionated into 2 parts containing: all pollutants and only polycyclic aromatic hydrocarbons. Dry residue of this fractions were dissolving in DMSO and tested using biological methods. Biological methods include mutagenicity properties which are investigated by *Salmonella* assay (Ames assay). Other biological method was comet assay and 4 parameter cytotoxicity test PAN-I assay. Results of the conducted experiments shows differences in mutagenic, genotoxic and cytotoxic properties between seasons of collection and between volume of dust pollutions fractions. The worst properties shows particles collected in autumn and winter season and this containing only polycyclic aromatics hydrocarbons. Results showed also some correlations in results obtained during different methods and properties.

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# 1 Introduction

There is more than 2,000 chemical substances in the ambient air. Some of them are in a gas form, the other are absorbed on the surface of the particulate matter. Among this ones which are absorbed on the surface of particulate matter there are inter alia polycyclic aromatic hydrocarbons, phenols, organic compounds and many more [1, 2].

Particulate matter with all absorbed substances can penetrate the human respiratory system may cause health problems among which it is worth mentioning asthma, pneumonia, decreased lung function and cardiovascular system. The smaller particulate matter can penetrate deeper parts of airways and reach directly into alveoli. All these factors cause health problems induced by dust pollutions are observed not only in groups exposed to environmental risk (children and elderly people) but also in health part of population [3, 4].

Recently in May 2016 the World Health Organization present the report with the average concentrations of particulate matter fraction 10 μm and 2.5 μm obtained in cities among the world. The data presented by WHO comes from local air quality monitoring centres. In a report appears many of Polish cites which are on the top the polluted regions in European Union. One of the mentioned cities is Wrocław – the urban and industrial agglomeration, where air pollutions comes from three main sources: low-emissions, industrial emissions and urban traffic. Although the air quality in our city was improved in recent years and it has been observed reduction in annual average concentrations of particulate matter, there are still happened smog situations especially in winter season. The biggest problem in particulate pollution in Wrocław agglomeration is low emission caused by low quality coal combusted for heating houses not connected to the central heating network [5].

This article presents results of biological tests carried out on dust particles collected in Wrocław agglomeration during all year long. Tests conducted during the research included mutagenicity *Salmonella* test, genotoxicity comet assay and cytotoxicity PAN-I assay.

# 2 Materials and methods

## 2.1 Particulate matter collection and extraction

Material for research was particulate matter air pollutions fraction 2.5 μm collected Wrocław agglomeration (Poland). In Wrocław is a capital city of Lower Silesia region in which lives about 630 thousand residents with average density of population 2.1 thousand people per square kilometre. Samples were collected using high-flow air aspirator DHA-80 manufactured by Digitel Enviro-sense with cooperation with Regional Inspectorate for Environmental Protection. Samples were collected on filters made of glass fiber, which was replaced every 24 hours. This operation allows to obtain one sample which representatives one day of collection. The collected particulate matter consisted fraction with a diameter between 2.5 μm and 0.01 μm. Samples were taken during all year long and was divided into 4 seasons: spring from April to June, Summer from July to September, Autumn from October to December and Winter from January to March.

The test area was located on the corner of Wiśniowa street and Powstańców Śląskich street in the city center, directly near one of the major routes in the southern part of Wrocław. In close neighborhood place of sampling are located single- and multi-family residential buildings, the majority of which are not connected to the municipal heating network. The heating of this buildings is carried out by individual source of heating.

Dust extraction was carried out in 16-hour cycle with a 15-minutes reflux in conditions of reduced light. As the extraction solvent was used dichloromethane in an amount of 350 $cm^3$ for each sample. After extraction samples were placed in a vacuum evaporator for concentrating and vaporization solvent to dryness [6, 7].

In addition, the dry extracts were fractionated to obtain clear fractions containing polycyclic aromatic hydrocarbons (PAH). This fraction with the starting extract gives material subjected to biological tests [7, 8].

## 2.2 A549 cell line

Cytotoxicity tests were performed on the A549 cell line which are adenocarcinomic human alveolar basal epithelial cells (American Type Culture Collection, Cell Culture Line 185). The choice of adherent A549 cells allows to stimulate conditions as close as in real ingress finest fractions of particulate matter pollutions to the alveoli. A549 cell line is widely used as an in vitro model for various studies, including cytotoxicity studies of air pollutions [9, 10].

Breeding of A549 cell line was carried out in culture medium Dulbeco containing 10% addiction of calf serum, 2mM L-glutamine, 100 units/$cm^3$ penicillin and 100 µg/$cm^3$ streptomycin. Cell culture was incubated at 37°C and 5% of carbon dioxide atmosphere.

## 2.3 Biological tests

### *2.3.1 Salmonella assay*

In the *Salmonella* assay was used two Salmonella typhimurium TA98 strain. This was a gift from K. Sugiyama and M. Yamada from Division of Genetics and Mutagenesis, National Institute of Health Sciences, Tokyo, Japan. Characteristic of TA98 strain allows him to check mutagens caused phase change read, which transform strain to form nonhistidinedependent.

Experiment was conducted in the absence and in the presence of microsomal fraction S9, obtained from liver of Wistar rats and activated with Aroclor 1254. The microsomal S9 fraction was used with the purpose of metabolic activation of promutagens. Protein content in the fraction, as determined by Lowry's method, was 64.44 mg/$cm^3$. S9-mix was used in experiments, and the S9 content in the S9-mix was 4% (v/v).

*Salmonella* assay was conducted in the KADO procedure. This procedure allows to save the volume of samples tested and volume of rat leaver microsomal fraction. Reducing the amount of input sample, while provided optimal results in this procedure allows preincubation in temperature of 37ºC and 90 min.

### *2.3.2 Comet assay*

Comet assay was performed using OxiSelect™ Comet Assay Kit from Cell Biolabs Inc. according to the methodology described in the instruction of use on A549 cell line. This test can be divided into three stages: exposure the cells for the particulate matter, cells immobilization in agar gel and electrophoresis with result read.

The study was performed using method of directly contact samples on cell line A549. On the 24-well plate was prepared cell culture in density 1 x $10^6$ cells/$cm^3$ incubated by 24 h. After this time on the plate was applied particulate matter sample with appropriate dilutions and leaved to incubation for 48 hours at temperature 37ºC and 5% carbon dioxide atmosphere. Assay was carried out in two replications for each concentration of dust.

Moreover on the plates was prepared positive and negative controls which were DMSO and phenol.

In second step after the incubation the cells were scraped from the vessel and resuspended with PBS without $Mg^{2+}$ and $Ca^{2+}$ solution to obtain a cell density level 1 x $10^5$ cells/$cm^3$. Cells were transferred to the slides and compared with agarose in 1:10 ratio.

Electrophoresis was performer in an alkaline solution at voltage of 23 V and 300 mA for a period of 30 min. Reading in the presence of Vista Green DNA Dye was made using epifluorescence microscope Nikon Eclipse 90i. For analysis of the results was used CASPLab program. It was observed not less than 100 comets in each sample.

### *2.3.3 PAN-I cytotoxicity test*

Cytotoxicity tests were performed using PAN-I assay containing four single tests to evaluate different parameters defining toxic effects on cells. Parameters measured by PAN-I assay was: integrity of cell membrane (LDH test), mitochondrial activity (XTT test), lysosomal activity (NR test) and total protein content (SRB test). All tests were performed on the same breeding of A549 cells in the 96-well plates. On the monolayer cell culture it was applied particulate matter fraction 2.5 μm extracts with appropriate dilutions. The exposure time was 48 hours. After that it was performed all toxicity tests starting from LDH according to the instruction of use. As a positive controls was used solvent (DMSO) and as a negative control Triton X-100.

Only LDH test was performed in the supernatant of the culture medium with using kinetic results reading by the time 1 hour at 22ºC and 2 min interval. Other tests were used on the original cells washed after each one with PBS solution. Results of the PAN-I assay was reading by the spectrophotometer μQuant with a specific wavelengths. The used wavelength was as follow: 340 nm for LDH test, 480 nm for XTT test and 540 nm for NR and SRB tests. In XTT, NR and SRB test it was also used reference wavelength of 690 nm.

## 3 Results and discussion

### 3.1 *Salmonella* assay

Mutagenicity of the air polluting particulates increases in heating season and decreases in summer [11]. As it has been proved, there is an inverse relationship between the mutagenicity and ambient temperature, and the maximum mutagenicity values are reached under winter conditions [12].

Results obtained in *Salmonella* assay are presented below. In case of TA98 strain no matter with S9 or not the potential mutagenicity samples were raw extract of pollutants labeled as “all” in the highest tested concentration of 25 $m^3/cm^3$. For this concentration the highest value of MR parameter (Mutagenicity Ratio) was 5.6 in case of autumn sample with S9 fraction, this sample reached also the highest value of MR with all tested samples with TA98 strain. The lowest potential mutagenicity for initial concentration was observed in summer samples tested with and without metabolic activation. The MR value in this cases reached 2.1. The value MR ≥ 2,0 was also obtained by samples of all pollutants in concentration 12.5 $m^3/cm^3$, but only examined with the absorbance of S9 fraction in spring – MR = 2.6 and winter MR = 3.0.

Samples collected during autumn season shows values of MR on the level 3.3 with metabolic activation and 2.0 without. In two instances the concentrations 6.25 $m^3/cm^3$ also have demonstrated the potential mutagenicity. This samples was for autumn and winter

with metabolic activation, which shows MR value 2.2. Other samples tested by TA98 strain did not exceed the rate of mutagenicity above 2.0, which can proves their mutagenicity character. Higher values of MR obtained samples collected during autumn and winter period compared to this collected in spring and summer.

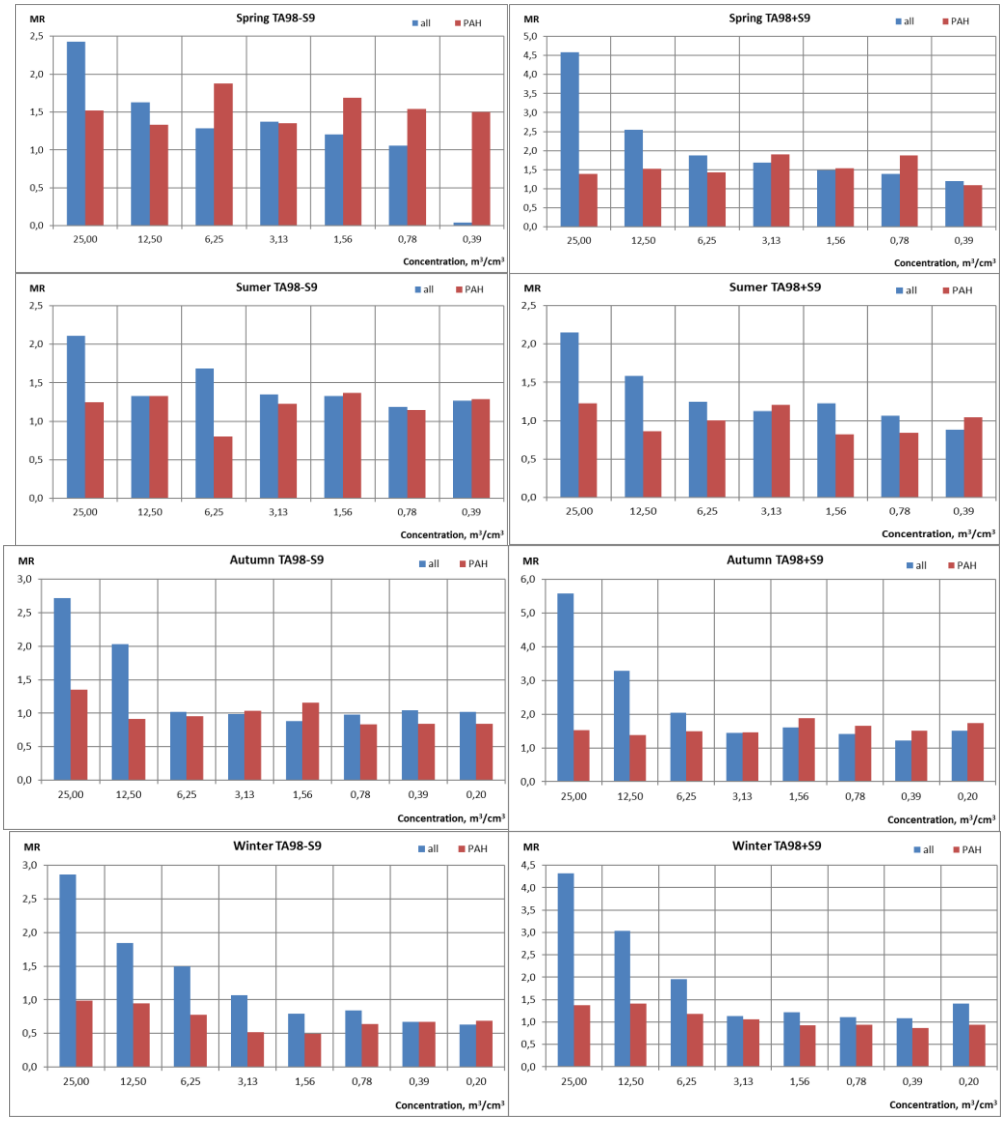


**Fig. 1.** Mutagenic Ratio of particulate matter fraction 2.5 µm extracts without S9 fraction (on the left) and with S9 fraction (on the right).

When analyzing samples containing only a PAH fraction it can be observed that none of them values higher than 2.0 of mutagenicity ratio. The highest value 1.9 was obtained for spring sample without metabolic in concentration 6.25 $m^3/cm^3$.

Claxton et al. (2004) reviewed results of examinations conducted on different parameter then mutagenic ratio. Comparing to this examinations performed in Europe showed in Czech Republic from 2 to 107 revertants/$m^3$ in winter, and from 2 to 23 revertants/$m^3$ in

summer, whereas in Germany, in densely populated, with heavy traffic, highly industrialized regions, noted were more than 100 revertants/m$^3$ of air [13].

### 3.2 Comet assay

The results of comet assay are presented as Tail Moment Length, which presents average distance from the center of the comet head to the center of its tail. It was measured at least 100 cells at each dilution of the test sample.

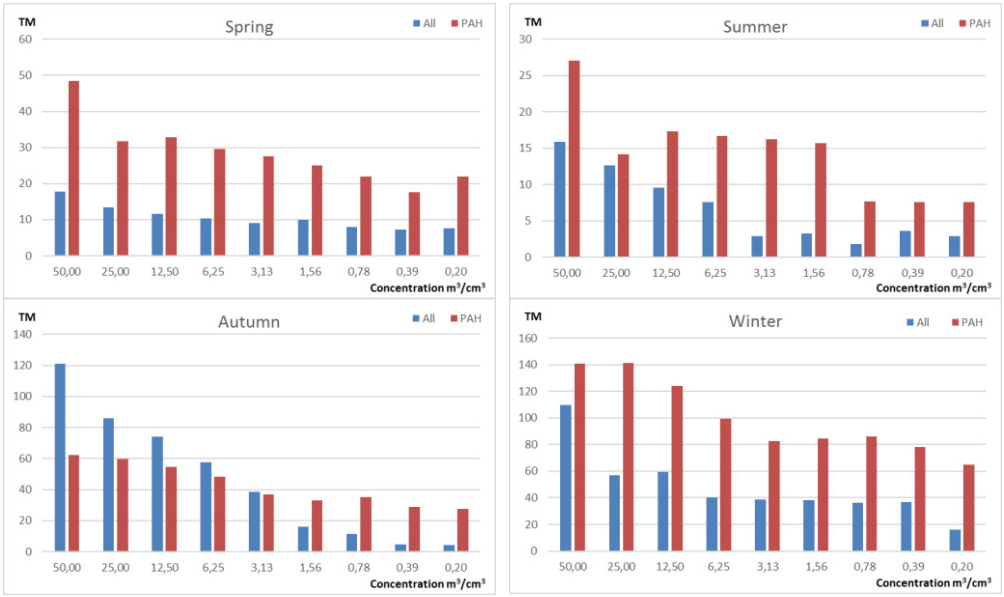


**Fig. 2.** Tail Moment parameter of particulate matter fraction 2.5 μm extracts.

Tail Moment Length for control samples (culture medium with 1% DMSO) was 20.972. The highest value of the Tail Moment Length parameter showed PAH fraction for winter season from 141.254 to 64.944, exceeding the level of control sample even in the lowest concentration tested. Genotoxic properties of particulate matter were also obtained for autumn and spring season. During summer months only the largest tested concentrations showed Tail Moment parameter above 20.972. In extracts containing all absorbed pollutants the highest genotoxic properties showed samples collected in autumn. Not much lower values were obtained for winter samples. Spring and summer values did not exceed control sample.

Research performed in Brazil on a V79 cell line shows various tail intensity. Tested concentration of 10 μg/cm$^3$ of organic extracts shows genotoxic effect of cells from 18.4 to 44.09 in winter-autumn season and form 7.20 to 36.13 in spring-summer season [14].

### 3.3 PAN-I cytotoxicity test

The results of cytotoxicity assays are shown as $EC_{50}$, which presents the concentration causing 50% inhibition of observed effect. The results are shown in the table below.

The results presented in the table show much higher values in the case of LDH test in comparison with the other three tests. This fact is result in disorders related to reading the samples containing color, which are undoubtedly particulate matter extracts, in this type of

test. In the other three toxicity testing obtained results were similar to each other, wherein it should be noted that the XTT assay result in most cases shows slightly higher than those obtained in the NR and SRB tests.

Lowest $EC_{50}$ values, which mean higher toxicity was showed for all pollutants sample collected during amount season. It was 2.810 for SRB test, 2.769 for NR test and 5.120 for XTT test.

**Table 1.** $EC_{50}$ concentrations of particulate matter extracts.

| Sample | | LDH | XTT | NR | SRB |
|---|---|---|---|---|---|
| Spring | All | 23.000 | 11.682 | 5.687 | 4.674 |
| | PAH | 24.000 | 11.926 | 5.270 | 6.340 |
| Summer | All | > 50.000 | 30.757 | 18.773 | 15.570 |
| | PAH | 25.000 | 34.642 | 22.906 | 18.668 |
| Autumn | All | 26.000 | 5.120 | 2.769 | 2.810 |
| | PAH | > 50.000 | 25.648 | 11.855 | 8.486 |
| Winter | All | 43.000 | 17.300 | 6.045 | 5.634 |
| | PAH | > 50.000 | 9.143 | 7.917 | 5.622 |

Low EC50 values are also obtained for winter and spring samples (both all and PAH fraction). The highest values which means lowest toxic properties showed samples collected during summer months from 15.570 in SRB test to 30.757 for XTT.

Impact of the particulate matter and organic extracts were also investigated by other authors. Among many results of researches it was measured apoptosis an viability of A549 cell line and cytotoxic effects using different parameters including LDH test, MTT test, which is alternative to the XTT, and MDH test [10, 15–17].

## 4 Conclusions

The presented results lead to the following conclusions:

1. It has been observed difference between on obtained results in various seasons of collection.
2. It has been proven worse properties of particulate matter collected during autumn-winter period in comparison to spring-summer ones.
3. *Salmonella* assay shows higher mutagenicity effect in all absorbed pollutions than in PAH fraction.
4. In comet assay only in autumn season all absorbed pollutants showed higher genotoxicity then PAH fraction. In other seasons higher tail moment parameter was observed in relation to PAH fraction.
5. It has been shown inaccuracy of LDH test in conducted formula. It is proposed to change the method of carrying out, to not read the results in supernatant.
6. It has been shown similarity the results of $EC_{50}$ obtained in the NR test and SRB test and lower sensitivity of the XTT test.
7. It has been confirmed effectiveness of using A549 cell line in researches of particulate matter fraction 2.5 μm.

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