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Original Research

Mutagenic Activity of Environmental Air Samples from the Area of Wrocław, Poland

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Abstract

It was found that the pollutants adsorbed on airborne particulates are a real threat to the health of inhabitants as high values of mutation ratio were obtained in the *Salmonella* plate incorporation assay (Ames test) for extracts of air samples collected during the summer and winter seasons in the centre of the town of Wrocław. The presence of pollutants which might directly and indirectly affect the genetic material has been indicated in tested samples. Mutagenicity of the air samples was highest in winter and lowest in summer. The studied samples collected in different seasons of the year were mutagenic to all tested strains. The *Salmonella typhimurium* strains: TA 98, YG 1041 and YG 1042 have been found most useful for detection of genotoxic air pollutants in urban agglomerations.

Keywords: air pollution, glass filters, the Ames *Salmonella*/microsomal assay, mutagenicity, polycyclic aromatic hydrocarbons

Introduction

Industrial processes, traffic, power generation, waste incineration and fuel or coal combustion for heating purposes are major sources of air pollution [1]. Emission of pollutants leads to their increasing concentration in the air and penetration to other elements of the environment (soil and water) and to living organisms. Air pollution is considered by the World Health Organization as one of the environmental exposure situations that can affect human health, contributing to acute respiratory infections, cancer, chronic respiratory and cardiovascular diseases [2].

Air pollutants constitute a complex mixture of various chemical compounds. Some of them are gaseous and some are adsorbed on airborne particulates, and together with dust particles may be inhaled. Recent epidemiological studies in the USA have revealed an association between excessive mortality and the complex mixture of fine airborne solids smaller than 10 μm, particularly those smaller than 2.5 μm. Furthermore, in the most polluted areas a remarkable increase of lung cancer cases has been observed [3-5].

Due to the limited self-purification capability of atmospheric air, it is necessary to take measures to protect it against excessive pollution based on monitoring air cleanness. The degree of air pollution can be assessed by determining the concentration of air pollutants and PAHs from the EPA list, and subsequently comparing those values with limits defined by law. Such a procedure, however, allows only for the assessment of current state of the environment and does not provide any data regarding the impact of pollution on living organisms [2]. Therefore, apart from analytical methods, it is necessary to apply bioindication tests to the control of the atmospheric environment [6].

Numerous investigations on the mutagenicity of ambient air samples dealt with the causal function of polycy-

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clic aromatic hydrocarbons (PAHs) and their nitro- derivatives (NPAHs) in observed mutagenic activity [7]. The particulates show direct and indirect mutagenic action, and NPAHs are considered the main cause of direct mutagencity, while PAHs contribute to the indirect mutagenicity. Several NPAHs such as 1- nitropyrene (NP), 1,3-, 1,6- and 1,8- dinitropyrenes (DNPs), and 2- nitrofluorene (2-NF) show very strong direct mutagenicity [8].

These chemicals cannot be tested directly on human subject, and even if such tests were possible, obtaining the results would take too long to be considered practicable. Mutagenicity of chemical compounds can be evaluated by quick methods that were developed over the years. They are usually based on reversion of certain features of genetically modified test organisms [9]. The *Salmonella* bacterial test was successfully used to detect mutagenic activity in air samples. The Ames *Salmonella*/microsomal assay uses bacteria as sensitive indicators of DNA damage, and mammalian liver extracts (S9 mix). The Salmonella assay with or without application of metabolic activation proved to be useful for the detection of both promutagens and direct-acting mutagens in airborne particulate matter collected from different cities of the world [10-16].

The aim of the current study was to determine whether the atmospheric air collected during the summer and winter seasons in the highly industrialized Wrocław area (Poland) contains pollutants positively responding to the Ames test.

Experimental Procedures

Collection and Preparation of Air Samples

Tested material consisted of airborne particulate matter occurring in the atmospheric air in the area of Wrocław. The samples were collected in the district Stare Miasto in Nowy Targ square, situated in the city center, near the main arterial streets of Wrocław.

Air samples were collected during the summer season, from May to September, and during the winter season (heating season), from November to March.

The samples were taken using a Staplex aspirator in a continuous manner for 17–24 hours. In total, air pollutants were collected for 960 hours in winter, and 1669 hours in summer. The air aspiration rate was 71.7-82.08 m^3/h. The air was aspirated through a Schleicher & Schull glass filter GF-9, pore size 0.5-1.4 µm. The total amount of filtered air was 122,326 m^3 in winter series of tests, and 161,004 m^3 in summer series of tests.

After collection of pollutants the filters were dried to constant weight and their mass was determined. The total mass of solid particles collected from winter samples was 7.07565 g, and from summer samples 7.0419 g.

Then the filters containing the collected solid particles were extracted with dichloromethane using the Soxlethe extractor, in the absence of light, for 8 hours, applying a 15-minute reflux [10, 17, 18]. Once the extraction was completed, dichloromethane was evaporated in a vacuum evaporator. The winter-sample extract and the summer-sample extract were then dissolved in 122 cm^3 and 161 cm^3 of dichloromethane, respectively. The 5 cm^3 samples of each of the solutions were again evaporated to dryness. Each of them was extracted with 10 cm^3 of acetonitrile while being exposed to ultrasounds for 1 hour, and then subjected the HPLC analysis for PAH presence. The HPLC analysis was performed on 10 µl samples, using a Varian chromatograph that offered spectrofluorometric detection, provided with a C18 stationary phase column. An acetonitrile-water mixture was used as a mobile phase at the following gradient: water 50% – acetonitrile 50% (v/v), 5 minutes; up to 100% acetonitrile – 45 minutes; water 50% – acetonitrile 50% (v/v), 10 minutes. Flow rate: 0.5 cm^3/min, separation temperature: 30°C. Individual PAH concentrations in samples were calculated from the regression equation, taking into account the size of analyzed samples and volume of air through the filter. The chromatographic results were reduced to a 1 cm^3 sample obtained from 1000 m^3 of air. The other dichloromethane solutions were re-evaporated on the evaporator and air-dried; dry residues of the winter-sample and the summer-sample were dissolved in 117 cm^3 of DMSO and 156 cm^3 of DMSO, respectively. The obtained solutions contained, per 1 cm^3 volume, contaminations originally occurring in 1000 m^3 of air. Such prepared samples were used in the Ames Assay.

Bacterial Strains

The criterion for selection of the test strains was their high susceptibility to the action of PAH mutagens, as well as their nitro, amino and hydroxylamine derivatives.

Four test strains were selected for detection of his^- to his^+ reverse mutation in our study: *Salmonella typhimurium* TA 98, TA 100, YG 1041 and YG 1042 (Table 1). The *Salmonella* test strains were obtained from Dr. T. Nohmi, Division of Genetics and Mutagenesis, National Institute of Hygienic Sciences, Tokyo, Japan.

Each test strain contains a different type mutation in the histidine operon (his^-) and other mutations that greatly increase their ability to detect mutagens, for example: rfa mutation linked to a partial loss of the lipopolysaccharide barrier, and uvrB is a deletion of a gene coding for

Table 1. *Salmonella typhimurium* strains used.

Strain	Description
TA 98	TA 1538 his D3052 (pKM101)
TA 100	TA 1535 his G46 (pKM101)
YG 1041	TA 98 (pYG233): a nitroreductase and O-acetyltransferase- overproducing strain
YG 1042	TA100(pYG233): a nitroreductase and O-acetyltransferase- overproducing strain

the DNA excision repair system. The TA 98 strains detect frameshift type mutagenicity, and the TA 100 strains detect the insertion of base pair substitutes (BPS type) [19]. The YG 1041 strain was derived from the TA 98 strain, and YG 1042 from TA 100. The *Salmonella typhimurium* YG 1041 and YG 1042 strains show higher nitroreductase and O-acetyltransferase activity encoded on plasmids. Therefore, they are more sensitive to PAH nitro-, amino- and hydroxylamino derivatives [20].

Mutagenicity Assay

Mutagenicity was measured by the Ames assay, following the author's instructions [19]. The cells used in the assay were cultured in soft agar containing a trace amount of histidine to promote growth of auxotrophic bacteria, and the assay was designed to detect reverse mutations from auxotrophic cells to histidine-independent prototrophic cells.

Genetic markers of the test strain and the degree of its spontaneous reversion were checked each time before testing the samples. Average numbers of revertants formed spontaneously were close to those given by Maron and Ames [19], and Hagiwara et al. [20]. The strain sensitivity check was based on a positive response and performed by exposing the bacteria to diagnostic mutagens; without the metabolic activation which would take place in the presence of the S9 fraction: 0.2 µg of 2,4,7-trinitro-9-fluorene per plate for the TA 98 strain; 0.5 µg of 4-nitroquinoline-N-oxide per plate for the TA 100 strain; 50 µg of 2,6-dinitrotoluene per plate for the YG 1041 strain, and 100 µg 2,4-dinitrotoluene per plate for the YG 1042 strain; and with the metabolic activation: 2.5 µg of 2- aminofluorene per plate for the TA 98, TA 100, YG 1041 strains, and 5 µg of 2- aminoantrhacene per plate for the YG 1042 strain.

The test was carried out with and without use of the rat-liver microsomal fraction (S9 mix), induced with Aroclor 1254, which promotes the transformation of promutagens into mutagens. The fraction was obtained by the methods developed by Maron and Ames [19]. The biuret method was chosen from among many others, reported by authors who apply protein content tests in their work [21] in order to determine the protein capacity in the fraction. The method is based on detection of peptide bonds, which are characteristic of proteins, and its application is especially recommended in research on the microsomal fraction. The protein content, as determined by the biuret method [21, 22], was 64.44 mg/ 1 cm^3.

Air samples (1000m^3/1cm^3) were diluted in DMSO in such a way that the quantity of extract used per plate corresponded respectively to: 100; 50; 25; 12.5; 6.25; 3.125; 1.56; 0.78; 0.39; 0.195; 0.097 and 0.0485 m^3 of the tested air. All tests were repeated five times.

Revertant colonies (his $^+$ revertants) were counted after incubation at 37°C for 72 h. The number of his^+ revertant colonies in each sample was determined as a mean value of the five plates. The results were expressed as a mutagenicity ratio (MR) – the ratio of the number of *Salmonella typhimurium* revertants grown in the presence of the tested sample to the number of spontaneously appeared revertants. The sample was considered mutagenic when MR ≥ 2.

Results and Discussion

The comparison of data concerning the samples of airborne particulates (Table 2) revealed a significant difference in the quantity of airborne particulate matter and extracted tar substances depending on whether the sample collection took place in winter or summer season. The quantity of atmospheric dust collected in winter season equalled 132% of that collected in the summer, whereas the quantity of tar substances from winter samples was equal to 177% of that from summer-collected samples. A similar relationship was observed by other researchers [10-16]. The concentration of airborne particulate matter as well as the concentration of organic pollutants adsorbed on the particulates are considerably higher in the winter than in the summer; also those concentrations are higher downtown than in the suburbs. Higher quantities of contaminations adsorb on the particulates in winter season. In the summer, when the temperatures are higher, part of pollution becomes volatile and semi-volatile.

Table 2. Comparison of data regarding the collection of study samples of air pollutants, their mass and mass of obtained extracts (tar substances).

Type of sample	Capacity of air sample [m^3]	Mass of pollutants [µg/m^3]	Mass of tar substances [µg/m^3]
Winter	122 326	57.8428	17.57
Summer	161 004	43.737	7.56

Table 3. Content of PAHs in extracts of air pollutants collected from 1 m^3 of air.

PAHs	Type of sample			
	Winter		Summer	
	ng	%	ng	%
Pyrene	2.398	21	0.1276	23.8
Benzo(a)antrhacene	1.9872	17.46	0.1076	20.12
Benzo(b)fluoranthene	1.6674	14.65	0.0776	14.51
Benzo(k)fluoranthene	1.9858	17.45	0.059	11.03
Benzo(a)pyrene	2.228	19.57	0.039	7.29
Indeno(1,2,3,c,d)pyrene	1.1158	9.8	0.124	23.19
TOTAL	11.3822	100	0.5348	100

In both winter- and summer-collected samples (Table 3) the same six PAHs from the list of standard aromatic hydrocarbons were detected. The total content of PAHs detected in winter samples was 11.38 ng/m^3, as compared with 0.53 ng/m^3 in summer samples. The content of the most hazardous hydrocarbon, which is benzo(a)pyren, was 2.23 and 0.04 ng/m^3, respectively. This indicates that the daily absorbed dose of benzo(a)pyrene per person in winter was 25.7 ng, and in summer 0.45 ng, (on the assumption that human daily intake of air is about 11 m^3) [23]. The comparison of the PAH content detected in samples from winter and summer series indicates over 20 times higher concentration of these compounds in winter-collected samples. Such differences result from higher emissions occurring in winter in urban agglomeration areas, generated by combustion processes for residential heating purposes, and from higher car engine emissions – as a consequence of the fuel consumption increased by a dozen or so percent in winter [2]. Similar relationships have been obtained for air samples collected in Wrocław during winter and summer seasons at another sampling station, located 1 km from the station presented in the paper [14].

Found quantities of particular PAHs are within the limits reported in the literature, and are very close to those detected in urban areas of Belgium and in Prague [10, 12]. The quantities are also indicative of possible mutagenic activity of obtained airborne particulate extracts, which can be concluded from numerous papers [10-16]. The temperature as well is a factor of great importance, determining the mutagenicity level of the airborne particulates. An inverse relationship has been found between the mutagenicity and the ambient temperature, where maximum mutagenicity is reached under winter conditions [2, 8]. This is also related to increased atmospheric inversion, due to which the polutants keep close to the ground, generated by the same intensified sedimentation of mutagenic matter on particulates during winter months. Changes are observed also in chemical processes in the atmosphere as a result of changed content of ozone and nitrogen oxides [24, 25].

Due to interactions between pollutants, both in the atmosphere and in living organisms, the chemical examinations do not fully reflect the genotoxic activity of air pollutants. Thus the mutagenic activity of a given sample depends on many factors: concentration of particulates in the air and the concentration and quantitative composition of the pollution adsorbed thereon. The biological effect (synergism) of a given sample will depend on its qualitative and quantitative composition and, in particular, on the proportion between specific chemical compounds adsorbed on the airborne particles.

The Ames test was carried out within the wide range of concentrations of the airborne particulate matter extracts, from 100 to 0.0485 m^3 of air per plate (Figs. 1-8). Owing to such a wide range of concentrations, in the case of the YG 1041 and YG 1042 strains it was possible to determine their mutagenically as well as toxically effective concentrations (an inflection point on a dose-reaction curve was observed). Within the range of tested concentrations a distinct curve of the dose-response relationship was observed for the mentioned strains, which demonstrates the biological effect of the pollutants present in the sample, in relation to their concentration. In the case of TA 98 and TA 100 strains even the concentration of pollution coming from 100 m^3 of air was not sufficient in order to determine toxicity thresholds for the examined samples. When examining extracts of airborne particulates in the presence of those strains, one should use a higher pollution dose (more concentrated sample) in the test, in order to determine an inflexion point on the dose--response curve.

The obtained results indicated the creation of a higher number of revertants (an thus higher MR values) in winter samples than in summer samples, which is consistent with results obtained by other researchers [10-16]. The maximum number of revertants for specific test strains was obtained for different concentrations of pollutants introduced in the tests, which suggests that individual strains differ regarding susceptibility to the studied compounds. Many different biologically active pollutants adsorb on airborne particulate matter. Their action may be either mutually enhanced or weakened. The response obtained from the Ames assay in the presence of different test strains is a result of all such interactions.

The highest MR values were noted for the YG 1041 strain (Figs. 5, 6). This strain evidently reacted to the pollutants present in the samples because high numbers of revertants were obtained in the tests conducted on samples containing small amounts of pollutants. In the case of this strain a clear linear correlation was observed between the sample concentration and the number of the revertants obtained until the point when the toxic effect occurred. The maximum MR value for this strain, in case of samples collected in winter and tested without the fraction, was 35.63 (6.25 m^3/plate), and respectively 46.97 (12.5 m^3/plate) when tested with the fraction. The highest MR values were observed at lower content of pollutants introduced in the tests than in samples collected in the summer. The mutagenicity ratio of 43.16 was obtained from tests conducted without the fraction on summer samples of concentrations twice higher than the winter samples (12.5 m^3), and as high as 62.72 from the tests with the fraction. Such results suggest that in the experiment where a geometric pattern of sample dilution was applied, the concentration of pollution at which the maximum mutagenic effect would occur, has been missed (Fig. 5). The lowest MR values were obtained for the concentration of 0.0485 m^3 of the air per plate. This indicates that at the above-mentioned concentration, mutations are not generated, except the test conducted without the fraction for the winter sample (MR=3.15).

For the TA 98 strain (Figs. 1, 2) lower MR values were obtained, compared with the YG 1041 strain. No toxic effects were detected for this strain within the tested range of concentrations. In the case of winter-collected samples

the MR values obtained for the TA98 strain were higher when tests were carried out without using the S9 fraction. In the case of the summer-collected sample the higher MR values were obtained in tests using the S9 fraction, although the differences between the MR values obtained with and without using the fraction were rather small. When no metabolic activation was applied, the mutagenic activity was found higher for the sample of particulate matter collected in winter, which is indicative of a significantly higher fraction of compounds, considered as direct mutagens, present in the winter sample in comparison to the summer one. According to many authors [2], the mutagenic effect of the airborne particulate matter can be attributed to more polar PAHs, i.e. their nitro- and oxy-derivatives. The PAH nitro-derivatives show properties of strong direct mutagens. Their presence was noted also in the Wrocław urban area, during both winter and summer seasons [26]. The lowest concentration of the winter sample, at which the mutagenic effect was noted in the test conducted on the TA98 strain without application of the fraction, was 1.56 m^3 per plate, and 3.12 m^3 per plate – if the fraction had been applied. The lowest concentration of extracts from the summer-collected airborne particulate matter, at which the mutagenic effect occurred, corresponded to the quantity of particulates collected from 12.5 m^3 of air.

In the case of the other two strains, TA 100 and YG 1042 (Figs. 3, 4 and 7, 8), much lower numbers of revertants were obtained, which indicates their low susceptibility to the studied pollutants, present in the tested samples.

In case of the TA 100 strain (Figs. 3, 4), the maximum number of revertants (MR = 5.68) was obtained in the test carried out without the fraction, for the sample containing pollutants from 50 m^3 of the air collected in the winter. A similar value (MR = 5.48) was obtained for the test conducted with the fraction, when introducing the pollution from as much as 100 m^3 of air. The MR values obtained for the samples collected in the winter season were almost twice higher than those for the samples collected in the summer. As one can see, at such high concentrations of air

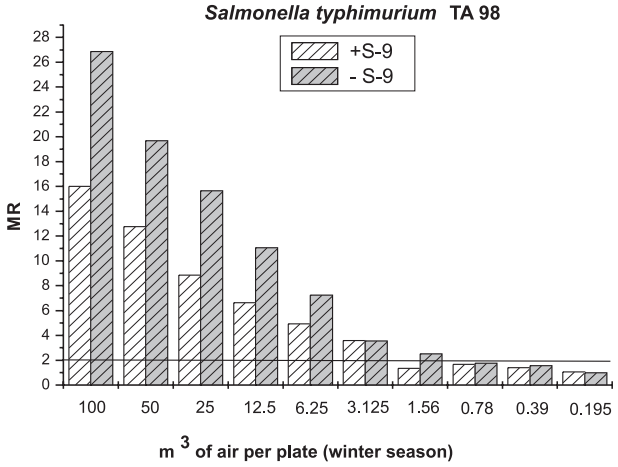

Fig. 1. MR values for the sample of air collected during the winter season.

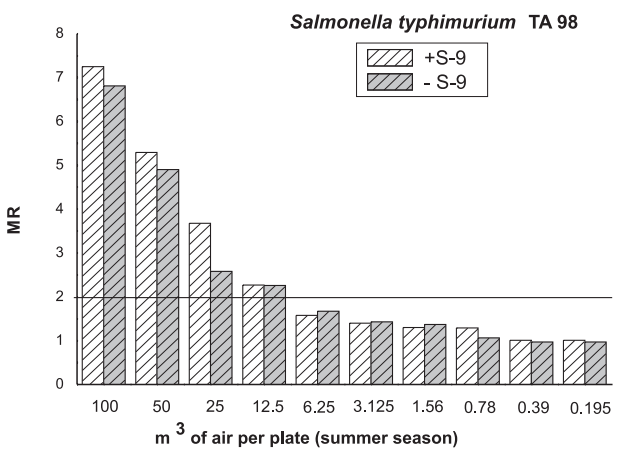

Fig. 2. MR values for the sample of air collected during the summer season.

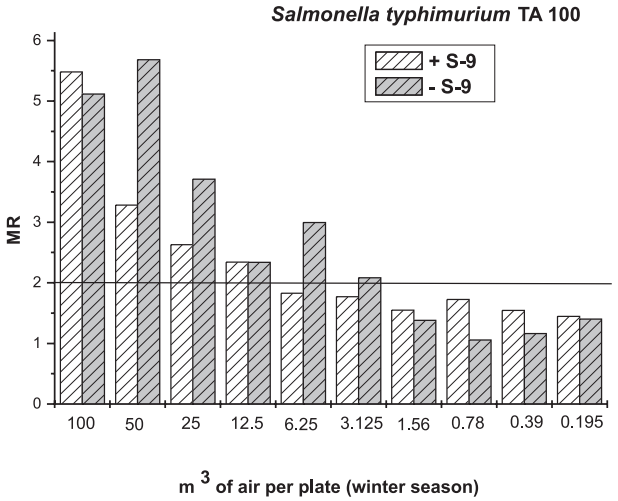

Fig. 3. MR values for the sample of air collected during the winter season.

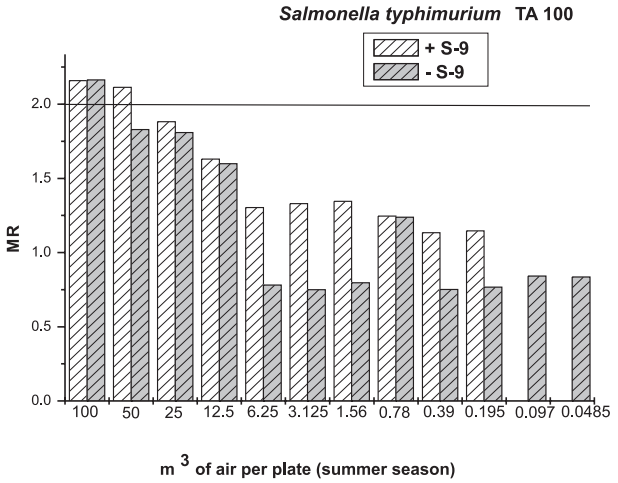

Fig. 4. MR values for the sample of air collected during the summer season.

pollution present in the test samples, still no toxic effects towards this strain were observed.

Still lower numbers of revertants were observed in the tests carried out for the YG 1042 strain (Figs. 7, 8), derived from the TA 100 strain. The maximum MR values found for the summer and winter samples in the tests carried out without the fraction were between 4.58 (3.125 m^3/plate) and 4.82 (25 m^3/plate). In the case of tests carried out with the fraction, the MR values ranged between 2.26 (6.25 m^3/plate) and 2.39 (25 m^3/plate). The lowest concentration of pollution tested for this strain (0.0485 m^3/plate) rendered the results corresponding to the spontaneous mutation.

Results and Disscusion

The tested samples of air pollution, collected in the area of the city of Wrocław, demonstrated a mutagenic activity toward the test strains of *Salmonella typhimurium,* namely TA 98, TA 100, YG 1041 and YG 1042, used in the Ames test. The samples of city air pollution collected in the winter demonstrated a higher mutagenic activity, compared with the samples collected in the summer, which is mainly linked to the heating season. High mutagenicity ratios (MR= 43.16 for the test without the fraction, and MR= 62.72 for the test with the fraction) obtained both for the tests carried out with the use of microsomal S9 fraction and without it, prove that pollutants which might affect the genetic material both indirectly (promutagens) and directly (direct mutagens) are present in the test samples.

The highest MR values for the *Salmonella typhimurium* YG 1041 strain were obtained in the tests carried out with the fraction S9. Thus this strain turned out to be the most susceptible to the promutagens present in the samples. Furthermore, very high MR values for this strain prove that nitro- and amino- PAH derivatives are present on the airborne particulates, as the strain shows higher nitroreductase- and O-acetyltransferase activity.

In the case of the *Salmonella typhimurium* TA 98 strain, high MR values were obtained for the most con-

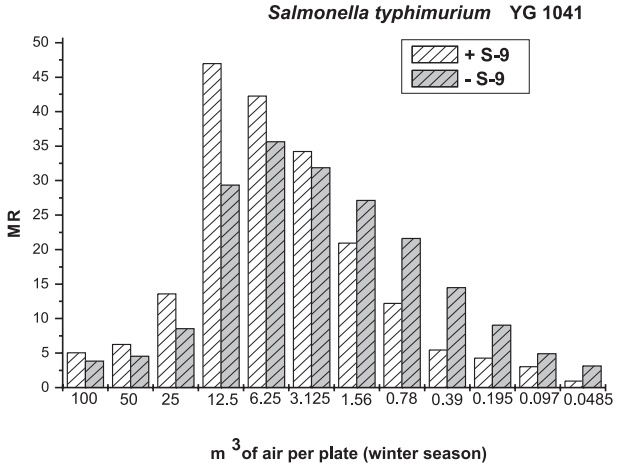

Fig. 5. MR values for the sample of air collected during the winter season.

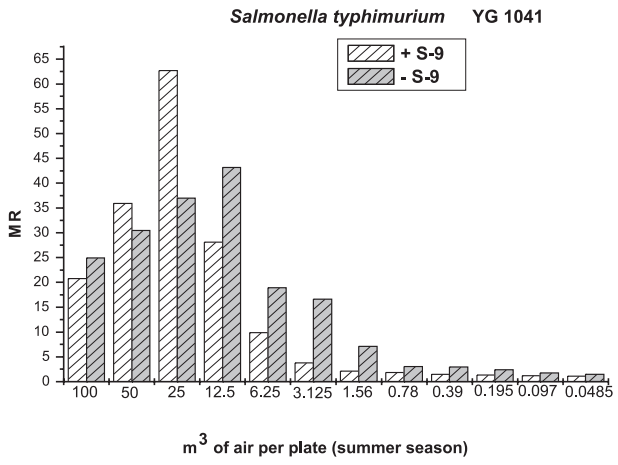

Fig. 6. MR values for the sample of air collected during the summer season.

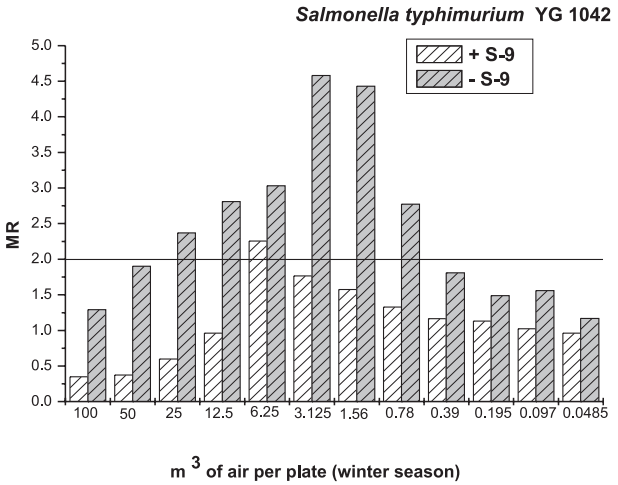

Fig. 7. MR values for the sample of air collected during the winter season.

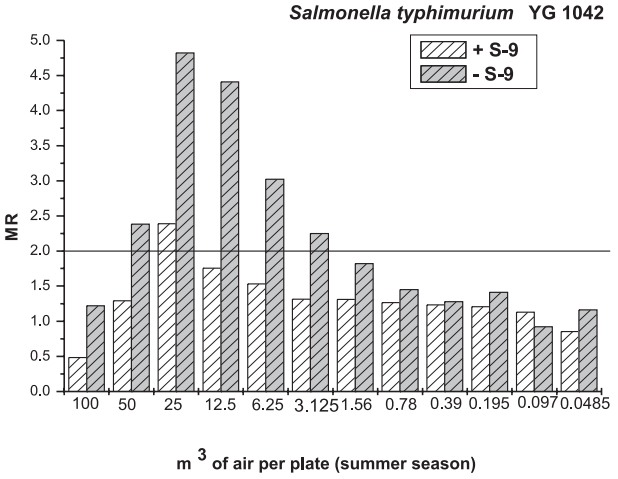

Fig. 8. MR values for the sample of air collected during the summer season.

centrated samples collected in the winter season. This means that the TA 98 strain responded to a higher degree to the direct mutagens.

Only minimal differences were found between mutagenicity ratios obtained in the tests conducted with the fraction and without it for the *Salmonella typhimurium* TA 100 strain, irrespective of whether the tested samples were collected in winter or summer. The ratio values were also low, which means that the strain would hardly be suitable for use in studies on the mutagenicity of air pollution.

The lowest MR values were obtained for the *Salmonella typhimurium* YG 1042 strain. Increased values of the mutagenicity ratio were noted for this strain at lower concentrations than in case of the TA 100 strain. The maximum MR values for the tests conducted with and without the fraction were obtained at much lower concentrations of the pollution introduced on the plate than in the case of the TA 100 strain. A similar relationship was observed for the TA 98 strain, when the tests were carried out without the microsomal fraction. Which means that the TA 1042 strain, despite presenting lower values of mutagenicity ratio compared to the TA 98 and TA 100 strains, can be helpful in detection of mutagenic substances at relatively low concentrations.

In conclusion of the tests it can be stated that the most useful strains for the detection of the genotoxic air pollutants in urban agglomerations by the Ames test are the *Salmonella typhimurium* strains: TA 98, YG 1041 and YG 1042.

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