Preliminary Microplate Ames MPF™ test use in assessment of mutagenic properties of spider webs

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Abstract. Air pollution is one of the most urgent problems of modern world, eventually causing the premature death of millions people every year. One of the burdens due to exposure to air pollutants is a lung cancer. It is necessary to develop new effective methods of carcinogen monitoring. The aim of the study was to evaluate if spider webs are suitable for the assessment of air mutagenicity by Ames MPF™ test. It is the first time spider webs had been incorporated into Ames MPF™ procedure. Webs of two spider species *Araneus diadematus* and *Agelena labyrinthica* have been collected at four sites exposed to high pollutants emission. *Salmonella typhimurium* TA98 strain without metabolic fraction have been used for the assay. All samples exhibited mutagenic activity most likely due to the road traffic. Webs of *A. labyrinthica* have shown higher mutagenicity effects at the tested sites in comparison to *A. diadematus,* plausibly because of the longer exposition time. The results are most promising and indicate high potential of combining spider webs and MPF™ procedure for assessing the mutagenic properties of urban air pollution.

1 Introduction

Air pollution is recognized to be a worldwide problem with significant threat to human health [1]. Pollutants emitted into atmosphere are mainly anthropogenic origin, deriving from transport, industry and households. In 2015 roughly 4.2 million people died due to outdoor urban and rural sources of pollution. The main causes of death were ischemic heart disease (17.1%), stroke (14.2%), lung cancer (16.5%), lower respiratory infections (24.7%), chronic obstructive pulmonary disease (27.1%) [2]. Air pollution, with particulate matter (PM) is a complex mixture of extremely small particles and liquid droplets that get into the air, was classified as carcinogenic by The International Agency for Research on Cancer [3].

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Polycyclic Aromatic Hydrocarbons (PAHs) and their nitro-, amino-, and other derivatives are especially harmful to human health due to their well-established mutagenic and carcinogenic characteristics and widespread prevalence. PAHs are formed mostly during incomplete combustion or pyrolysis of organic material [4, 5]. A significant source of toxic PAHs emissions are diesel engines [6].

Constant development of industry and increasing concentration of people population in big cities imposes new, more efficient and low-cost methods of monitoring of air pollutants, especially carcinogens. Chemical studies of air pollutants are not always effective for toxicity assessment. Contaminants properties might change due to their complex interaction with other chemicals and individuals as environment plays a crucial role in their activation [7]. Among many bacterial mutagenicity assays, the Ames test is most commonly used because of its effectiveness and relative simplicity. It is also recommended by International Agency for Research on Cancer. The tester strains of bacteria are designed to detect either frameshift (e.g. TA98) or point mutations in the genes responsible for synthesis of histidine, thus mutagens acting via different mechanisms may be identified [8]. Mutagenicity of dust suspended in the air have been assessed multiple times by Ames test all over the world [7, 9]. The new MPF-microplate format protocol for the evaluation of the mutagenic activity has been introduced in the commercial market recently which is gaining the popularity among scientists.

For studies of mutagenicity of air dust samplers such as impactors or passive samplers are usually used. However, classic application of impactors is limited. Such devices are expensive and can be used only for short-term studies. Moreover, passive samplers utilize selective sorbents which cannot be used for wide range of air pollutants. Thus, the development of alternative methods is promising. A successful alternative for such samplers is application of biomonitors due to their numerous advantages. Spider webs are a new tool particularly useful for biomonitoring of air pollutants, as they are nonselective, ubiquitous, costless and very able for long-term air monitoring.

The Ames mutagenicity assay based on spider webs has been applied for the first time in presented studies. The aim of the study was to test if webs with pollutants deposited on their surface are suitable for the assessment of mutagenicity by Ames microplate format test.

2 Material and methods

2.1 Spiders and webs description

Webs of two spider species *Araneus diadematus* from Araneidae family and *Agelena labyrinthica* from Agelenidae family have been chosen for the test. These families exhibit distinct hunting techniques and, what is the most important, are known from highly different web characteristics.

Araneidae, known as a common orb weavers, are among best recognizable and numerous spider families [10]. Significantly larger females spin vertical and sticky orb-webs. They usually face down on the centre of web, or remain hidden waiting for prey in nearby leafage. They are known for eating and rebuilding their webs in the morning or evening, depending on the species diurnal or nocturnal nature. Many orb-weaving spiders respond to factors like light or wind by modifying the orientation and/or structure of their webs during construction [11]. Araneids occupy wide range of habitats, including gardens, meadows, hedgerows. In urban areas they can be found next to buildings with exterior lighting, or between bridge spans.

Agelenids prefer to dwell in dark and neglected homesteads, especially in basements, attics, parking lots. They can be vastly found in road tunnels and under bridges. Spiders of

this family are commonly named the funnel weavers. They build non-sticky, horizontal webs with a funnel retreat on, or above the ground. Agelenids do not eat their large and dense webs. This features proved to be highly useful in monitoring of air pollution in a case of heavy metals and PAHs [12–16].

2.2 Sampling sites

Samples for mutagenicity test were collected between 3–8 of August 2017 from four sites (S1–S4) within city of Wrocław, Lower Silesia, Poland. Webs of *Araneus diadematus* were gathered from sites S1 and S3, *Agelena labyrinthica* webs, on the other hand, from sites S2 and S4.

S1 and S2 are placed fairly closed (within 120 m.), whereas S3 and S4 lie in the same area, in a distance of 650 m.

Site 1. 51°06′34.2″ N, 17°03′ 9.36″ E Grunwaldzki Bridge – a suspension bridge over the river Oder. This is one of the main road junction of Wrocław with a high-intensity car traffic 24 h a day. *A. diadematus* silk was collected from bridge spans. Webs were directly and constantly exposed to car exhausts. Since no service and maintaining works at the bridge have been done during that season, it can be assumed that spiders have not been bothered and dwelled there since May/June. However, as it was mentioned, orb-web spiders eat and rebuild their webs on a daily basis, so it was not possible to define the exact time of exposition.

Site 2. 51°06'34.8"N, 17°03'18.3"E Samples of *A. labyrinthica* webs were acquired from a low municipal greenery on Grunwaldzki square. This site is also located in a direct neighbourhood of jammed road traffic and car parking. Time of exposition to pollutants was approximately 6 weeks.

Site 3. 51°06′29″N 17°04′12″E Zwierzyniecki Bridge located in the eastern part of the town. The bridge spans the Oder River and lies in the moderate traffic street with an immediate vicinity of green areas and Wrocław Zoo. Samples of *A. diadematus* webs were also collected from bridge spans. Time of exposition is assumed to be similar to S1, but encounters the same difficulties.

Site 4. 51°06'34.6"N 17°04'44.9"E Samples of *A. labyrinthica* webs were obtained from low municipal greenery located nearby moderate traffic road and public facility Centennial Hall, which hosts every year major exhibitions, conferences, cultural, sport and congress events. Part of the facility consists of the parking lot, which serves for mentioned purposes. Webs were partially enclosed by a surrounding greenery. Time of exposition was evaluated for approximately two months.

Fig. 1. The map of study area.

2.3 Ames microplate format (MPF™) test

The Ames test was initially developed using agar plates but over time, novel, microplate test method has been developed. The principle of this modified method is consistent with agar plates assay, but is performed completely in liquid culture and is accomplished by counting the number of wells that turn yellow from purple in 96-well or 384-well microplates. Microplate test is acknowledged to be a reliable predictive tool which can be used like the regular Ames test to evaluate compounds for mutagenicity [17].

Salmonella typhimurium TA98 strain was possessed from Dr. T. Nohmi, Division of Genetics and Mutagenesis, National Institute of Hygienic Sciences, Tokyo, Japan. Reverse mutation to the wild-type state in TA98 strain can be induced by various frameshift mutagens [18]. Remaining materials essential for the test were included in test kit received from Xenometrix by Endotell.

2.4 Test procedure

Spider webs were assembled with glass rods and stored in dark bottles. Extraction with dichloromethane was performed in the dark for 48h. Each bottle containing sample was filled with 5 ml of dichloromethane, then solvent was allowed to evaporate. Procedure was repeated three times. Afterwards, the residues remained in bottles were resuspended in DMSO (1ml/20mg of web). Extracts of two-fold serial dilutions in DMSO were applied into the test kit. Test bacteria were exposed to 80 µl of six concentrations (20; 10; 5; 2.5; 1.25; 0.63 mg/ml) of each sample for 90 minutes in 24-well exposure plates. Triplicate exposition was performed in 37°C, on orbital shaker set at 250 rpm. Prior to the exposition, respectively: 10 µl of sample solution in DMSO and 240 µl of overnight culture without S9 fraction (-S9) had been added to each well of 24-well plates. After the exposition, 2.8 ml of indicator medium was added to each well of 24-well plates. Then, 50 µl aliquots of exposure mixture from 24-well plates were dispensed into 48 wells of 384-well plates. Next, 384-well plates were incubated for 48h in 37°C on orbital shaker set at 250 rpm. After this time, number of wells containing revertant colonies were counted for each dose.

Wells were considered positive if turned yellow or had bacteria colony visible on the bottom of the well. The fold increases above the baseline for each concentration was calculated as mean of negative control plus 1 SD. A baseline value was preferred to the negative control value during evaluation of MPF test, because it allows for better identification of true positive results in a system with very low spontaneous revertant counts and corresponding relatively high standard deviations [17].

Results of the test were estimated positive if number of wells containing revertants was at least 2-fold higher than negative control or 3-fold higher than baseline. Statistical analysis was performed using Excel sheet attached by test producer. Statistical significance of fold inductions in revertant numbers over negative controls were analysed by 1-sided, unpaired Student's t-test. Results were considered statistically significant when $p \leq 0.05$. By the procedure, results were claimed credible if mean number of positive wells (with revertants) in negative control did not exceed 8 and equalled at least 25 in case of positive control.

3 Results and discussion

All collected samples exhibit mutagenic activity for *Salmonella typhimurium* TA 98 strain (Table 1, Fig. 1). It implies, that direct frame shift mutagens have been detected on spider webs.

Spider webs collected from S4 (*A. labyrinthica*) display highest mutagenic properties with a range of four concentrations (2.11; 2.54; 3.66; 3.10 fold increase over baseline and 3.75; 4.5; 6.5; 5.5 fold increase over zero value for 2.5; 5; 10; 20 mg respectively).

Site 2 Webs of *A. labyrinthica* manifest high fold increase over baseline for 10 and 20 mg (3.1 fold for both concentrations) and are considered to be second most genotoxic.

In case of site 1 webs (20 mg) of *A. diadematus* display the highest fold increase over baseline noted in test (3.8 fold), with a mean of 9 positive wells, but at the same time, it is affected with high standard deviation of well number (SD = 2.8). At the same site, fold increases over baseline for 10 mg equals 2.25. Considering standard deviation and lower or equal fold increase over baseline for each lower concentration in comparison to S2 (0.85; 0.85; 0.85; 1.55 against 1.41; 1.41; 1.55; 1.55 respectively), Webs collected at S1 were found to be less genotoxic than webs at S2 and S4 (*A. labyrinthica* sites).

Webs of *A. diadematus* collected at site 3 exhibit lowest values of fold increase over baseline among all sites (2.1 and 1.9 per 10 and 20 mg respectively) and display lowest genotoxicity properties.

Table 1. Results of Ames test. SD = standard deviation,

Site	mg of web / ml	mean no. of positive wells	SD	Fold increase (over zero value)	Fold increase (over baseline)	t-test	Site	mg of web / ml	mean no. of positive wells	SD	Fold increase (over zero value)	Fold increase (over baseline)	t-test
S1	0	1.3	1				**S3**	0	2	1.2			
	0.6	2	1	1.5	0.8	0.19		0.6	2	1	1	0.8	0.2
	1.3	2	1	1.5	0.8	0.19		1.2	2.6	0.5	1.3	1.1	**0.04**
	2.5	2	1	1.5	0,8	0.19		2.5	3	2	1.5	1.2	0.06
	5	3.6	1.1	2.7	1,5	**0.008**		5	1.3	1.5	0.6	0.5	0.50
	10	5.3	2.5	**4**	**2.2**	**0.004**		10	5	2	2.5	**2.1**	**0.003**
	20	9	2.8	**6.7**	**3.8**	**0.0004**		20	4.6	1.5	2.3	**1.9**	**0.002**
	Pos. ctr.	32.7	6.51					Pos. ctr.	27.3	1.5			
S2	0	2	1.2				**S4**	0	1.3	1			
	0.6	3.3	2	1.6	1.4	**0.04**		0.6	2.6	2	2	1.1	0.11
	1.3	3.3	2.5	1.6	1.4	0.06		1.2	3.6	0.5	2.7	1.5	**0.004**
	2,5	3.6	1.5	1.8	1.5	**0.01**		2.5	5	1.7	**3.7**	**2.1**	**0.002**
	5	3.6	0.5	1.8	1.5	**0.004**		5	6	4.3	4.5	2.5	**0.01**
	10	7.3	1.5	**3.6**	**3.1**	**0.0001**		10	8.6	2	**6.5**	**3.6**	**0.0001**
	20	7.3	2	**3.6**	**3.1**	**0.0003**		20	7.3	0.5	**5.5**	**3.1**	**0.0000**
	Pos. ctr.	27.3	1.5					Pos. ctr.	31.3	5.6			

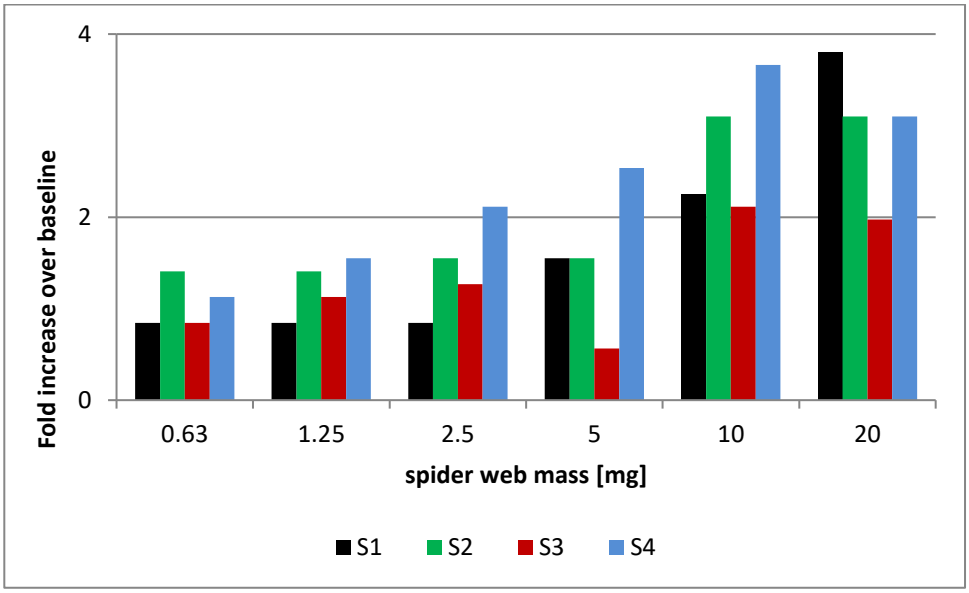

Fig. 2. Mutagenic activity of tested webs at sites 1-4 in the assay with TA98 strain without metabolic activation.

This is the first ever preliminary attempt to use MPF procedure for study of mutagenicity of spider webs. According to obtained results all studied sites were clearly mutagenic in the absence of metabolic activation (the variant without metabolic activation was conducted only). The MPF procedure is less laborious (e.g. all-liquid format, use of multichannel pipettors), therefore allows reducing time of the analyses which is particularly valuable in the fast air quality monitoring studies [19]. The application of MPF procedure on spider webs is particularly promising as it delivers cheaper, faster and better tool for air quality assessment comparing to conventional methods.

The results showed that all sites can be considered mutagenic probably due to the traffic – oriented localisation of sites (webs). Webs of *A. labyrinthica* have shown higher mutagenicity effects at both sites (sites 2, 4) in comparison to *A. diadematus* (sites 1,3). Although, mutagenicity effect was also observed for webs of *A. diadematus*. Sites 1 and 2 were located close to each other, but exposition time of collected webs differed. Webs of *A. labyrinthica* are woven again only when completely destroyed (in our study they were exposed much longer e.g. 6 weeks). Conversely, the webs of *A. diadematus* may be rebuilt every day, and the old web is consumed so that the proteins used in its construction are conserved and re-used [11]. The contaminants from the webs can be accumulated in the body of spider. Therefore, the level of web contamination of *A. diadematus* could be hypothetically higher as the proportion of pollutants found in orb-weaving spiders may reflect both atmospheric and soil levels. On the other hand, webs of *A. diadematus* were exposed significantly shorter to pollutants comparing to webs of *A. labyrinthica* (few days versus few months). This is probably the best explanation for the observed differences in mutagenicity at sites 1 and 2 and it could also explain why webs collected at site 4 were found the most mutagenic. At site 4 we collected the webs of *A. labyrinthica* which were exposed to pollutants from the beginning of vegetative season (approximately 2 months). Furthermore, the effect of meteorological conditions, in particular wind direction and speed, on pollutant concentrations should also be considered. Therefore, the influence of wind on traffic-related air pollutants should be also considered at site 4. Wrocław is

dominated by west winds, which influence the pollutants concentration, therefore site 4 could be more polluted even though this site is not very traffic oriented comparing to other sites. Although webs of *A. diadematus* were collected at highly traffic-oriented site (site 3) they exhibit lower level of mutagenicity due to particular behaviour of species (web eating) and therefore shorter exposure time.

The results presented in this work indicate the high potential of application both spider webs and MPF procedure for environmental studies especially in traffic-oriented sites localised in big cities polluted by both anthropogenic and natural airborne particulate matters, which may lead to negative health effects in human.

4 Conclusions

The aim of this study was to analyse if spider webs could be applied in evaluation of mutagenic effects induced by street dust using the Ames MPF procedure. The results show that the MPF procedure is a promising tool to fast testing of environmental samples for mutagenic activity. The study also demonstrated that spider webs are very useful for assessing the mutagenic properties of dust pollution, which exhibit toxic effect on living organisms. The use of microplate Ames assay together with application of spider webs seems to be an interesting alternative to the conventional studies.

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