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APPLICATION OF BIOASSAY WITH *SELENASTRUM CAPRICORNUTUM* TO EVALUATION OF TOXICITY OF ANTHRACENE AND PHENANTHRENE IN WATER

Polycyclic aromatic hydrocarbons (PAHs) belong to pollutants found in waters used as sources of drinking water. PAHs undergo microbiological transformation and photodegradation in environment. This may lead to alteration of toxic properties of the derivatives formed. Therefore it was important to investigate the influence of anthracene and phenanthrene (as model PAHs) and the mixture of their biodegradation metabolites on aquatic organisms exemplified by *Selenastrum capricornutum*. The test was performed in two ways on a mini- and macro-scales to evaluate its usefulness for controlling the state of fresh water environment. A mini-scale small volume of samples facilitated controlling and stabilising pH and temperature. Samples were also more effectively illuminated and mixed. The results obtained indicate that the mini-scale test is more reliable, more cost-effective and less time-consuming than the macro-scale test.

1. INTRODUCTION

Polycyclic aromatic hydrocarbons occur in all elements of environment: air, soil, natural water reservoirs, drinking water, plants, and also in food. They come from both natural and anthropogenic sources. However, the natural background is insignificant compared to the quantity of PAHs resulting from human activity and industry [1]. Particularly hazardous are toxic pollutants, which possess also mutagenic and carcinogenic properties, present in waters used as sources of drinking water. Concentration of PAHs, irrespective of their source, in surface waters should not exceed 150 ng/dm^3 [2].

In natural environment PAHs are biodegraded, photooxidized and ozonodegraded. The metabolites may differ in their toxicological characteristics from the substrates.

Predicting a biological activity of complex mixtures of chemicals is difficult, since it can be altered by small structural changes and by the synergistic or antagonistic effect of the substances present in environment [3].

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Toxicological bioassays allow us to evaluate the influence of mixtures of compounds on living organisms, they complement chemical analyses. Biotests are broadly applied to examination of water, soil and air as well as to identification of organisms, which are most at risk [3].

Algae belong to the first level of aquatic food chain, therefore pollutants cumulated in their cells are brought to circulation in the ecosystem. These organisms limit productivity of the ecosystem. Growth of their population can be inhibited or stimulated by the presence of pollutants, therefore they are important bioindicators of changes occurring in natural environment [4].

Algal assays are quick and effective. They are used to screen samples in order to identify pollution or to establish toxic profiles of chemical compounds [5], [6]. Such assays usually use green algae, particularly *Selenastrum capricornutum* from the order of *Protococcales* [7]. This species has become a "laboratory mouse" because it is easy to identify and culture and its cells undergo only minor morphological changes in the lifetime [8].

The ability of chlorophyll a to fluorescence allows us to estimate biomass of phytoplankton or synthesis of photosynthetic pigments. This method is sensitive, easy for handling (without the laborious extraction procedure) and gives reliable results well correlated with culture growth and cell number. *In vivo* measurements substantially reduce the time and sample volume required to perform the test [6], [9].

The aim of the present paper was to estimate the toxicity of model PAHs and their biodegradation metabolites for the producers of aquatic ecosystems. Further, we compared the mini-scale toxicological assay with *Selenastrum capricornutum* with a macro-scale test equivalent to the ISO Standard 8692.

2. EXPERIMENTAL PROCEDURES

2.1. MODEL COMPOUNDS

The study was carried out with two tricyclic PAHs, i.e. anthracene and its angular isomer phenanthrene, at the following concentrations: $5 \cdot 10^{-8}$, $1 \cdot 10^{-7}$, $5 \cdot 10^{-7}$, $5 \cdot 10^{-6}$ mole/dm^3. The PAHs were added to medium in 0.1 cm^3 of pure acetone.

Products of biodegradation of anthracene and phenanthrene by the strains of *Arthrobacter* sp. (4_1') and *Bacillus alvei* (16A) and by the mixed culture of bacteria were extracted from 750 cm^3 of mineral growth medium containing 5% glucose as a source of carbon as well as $5 \cdot 10^{-6}$ mole/dm^3 of anthracene or phenanthrene and $5 \cdot 10^{-6}$ mole/dm^3 of PAHs as sole source of carbon. Dry residues obtained after evaporation of extracts were dissolved in 1 cm^3 of DMSO and used in further experiments.

The following products of anthracene and phenanthrene degradation were identified: anthracene, 9,10-dihydro-,9(10)-anthracenone, 9,10-anthracenedion, phthalic anhydride, esters of 1,2-benzen-dicarboxy-acid, methyl derivatives of naphthalene and

esters of phthalic acid [10], [11]. Metabolites of the first phase of phenanthrene biodegradation were not identified. Chromatograms were also rich in low-intensity peaks representing fatty acids, aldehydes and alcohols with chain lengths varying from C-5 to C-16 which were natural products of bacteria metabolism and did not arise from PAHs degradation [10].

The following symbols were used:

Control– control sample;
4_1' – metabolites produced by *Arthrobacter* sp. (4_1');
16A – metabolites produced by *Bacillus alvei* (16A);
PM – metabolites produced by the mixed bacterial culture;
F+G – cometabolites of phenanthrene and glucose produced by microorganisms;
F – metabolites of phenanthrene produced by microorganisms;
A+G – cometabolites of anthracene and glucose produced by microorganisms;
A – metabolites of anthracene produced by microorganisms.

2.2. MODEL STRAINS

Highly active strains of *Bacillus alvei* (16A) and *Arthrobacter* sp. (4_1') were used in the experiments.

Based on earlier studies of the ability of bacteria to degrade PAHs and of antibiotic interactions between the strains of interest, the optimum mixed culture of bacteria was selected, which was composed of the following strains: *Bacillus thiaminolyticus*-14, *Bacillus circulans*-17, *Bacillus laevolacticus*-18, *Arthrobacter* sp.-21, *Pseudomonas* sp.-23, *Arthrobacter* sp.-4_1', *Bacillus alvei*-16A [11].

In order to obtain high activity, single strains and the mixed culture were gradually adapted according to a procedure given below. In the first week glucose was the source of carbon in the medium, and in the second week bacteria were transferred to the medium containing glucose with the addition of anthracene or phenanthrene. At the beginning of the third week bacteria were transferred to the medium containing one of the hydrocarbons as the sole source of carbon and energy [10].

2.3. *SELENASTRUM CAPRICORNUTUM* ASSAYS

Selenastrum capricornutum strain received from the United States Environmental Protection Agency was passaged and proliferated. Inoculum of *Selenastrum capricornutum* was prepared in a sufficient amount to ensure the concentration of algal cells in the test sample not lower than 10 000 cells/cm^3 [5], [6], [12]. The dependence of extinction of algae suspension on cell concentration (standardisation curve, figure 1) was determined. Algal cells were counted in the Fuchs–Rosenthal cell. Optical density of the culture was measured at 652 nm in spectrophotometer UV-1202 Shimadzu.

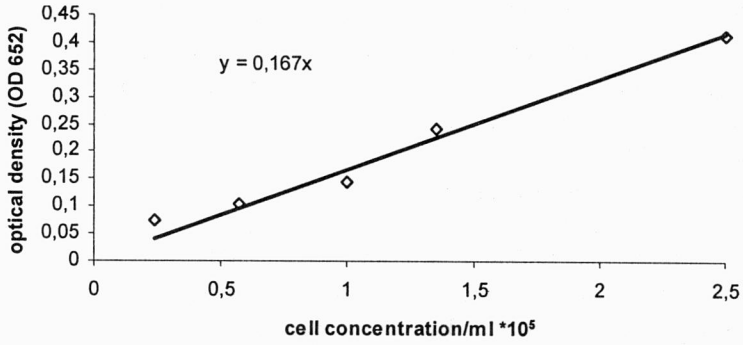

Fig. 1. The dependence of optical density (OD) of algae suspension on cell concentration (standardisation curve)

2.4. MACRO-SCALE TEST

Selenastrum capricornutum population was exposed, on the recommendation of the authors [5], [6], for 96 hours to chemicals at the following concentrations: $5 \cdot 10^{-8}$, $1 \cdot 10^{-7}$, $5 \cdot 10^{-7}$, $1 \cdot 10^{-6}$, $5 \cdot 10^{-6}$ mole/dm^3. The test was performed at a constant temperature of 24 °C, with cyclic illumination (4300 lux). Fluctuations of pH, ambient temperature and optical density were monitored throughout the test. Response of the population was expressed as changes in the concentration of chlorophyll a, which was measured by determination of its fluorescence (in fluorometer Perkin Elmer MPF-44, activating wave – 652 nm, emitted wave – 600 nm). This procedure allows us to determine the concentration of chlorophyll a *in vivo* [6], [13]. The metabolites were not used in the experiments because it was impossible to receive enough test material.

2.5. MINI-SCALE TEST

Algae were exposed for 48 hours to chemicals at concentrations of $5 \cdot 10^{-8}$, $1 \cdot 10^{-7}$, $5 \cdot 10^{-7}$, $1 \cdot 10^{-6}$, $5 \cdot 10^{-6}$ mole/dm^3 and to biodegradation metabolites applied as follows: $5 \cdot 10^{-3}$ cm^3 of metabolites dissolved in DMSO were added to 5 cm^3 of culture medium. This was the concentration of biodegradation metabolites (at initial concentration of $5 \cdot 10^{6}$ mole/dm^3), which were metabolised by microorganisms. The test was carried out at a constant temperature of 24 °C, with cyclic illumination (4300 lux). Changes of pH, temperature and absorbance were measured during the test [5], [6]. Response of the population was expressed as the changes of the concentration of chlorophyll a, which was measured by determining its fluorescence. This procedure allows us to determine the concentration of chlorophyll a *in vivo*. In this test stimulation is occasionally observed. The SC_{20} value is used most often to report stimulating effects. It represents the concentration of PAHs tested that results in a 20% increase in biomass

relative to that for the control algal population. The importance of stimulation in the data interpretation and definition of biologically relevant level should be estimated [14].

2.6. COMPARISON OF MACRO- AND MINI-SCALE TESTS

To compare the usability of the methods, the assay was performed on macro- and mini-scales (table 1).

Table 1

Comparison of macro- and mini-scale tests

Feature	Mini-scale test	Macro-scale test
Sample volume	5 cm^3	100 cm^3
Exposure time	48 hours	96 hours
Mixing	cyclic	cyclic
Illumination	4300 lux	4300 lux
pH fluctuations	± 0.1 unit	± 0.4 units

3. RESULTS AND DISCUSSION

The compounds studied, i.e. anthracene and phenanthrene, strongly influenced synthesis of chlorophyll a in *Selenastrum capricornutum* cells as shown by both mini- and macro-scale assays. In the mini-scale test, the increase of fluorescence was better correlated with population growth.

In the macro-scale test, pure compounds insignificantly influenced the changes in the fluorescence of the organism cells tested (figure 2). Stimulation was observed only in the case of anthracene concentration reaching $5 \cdot 10^{-7}$ mole/dm^3, where the SC_{20} was noted (the parameter tested increased by 20% in comparison with the control sample).

In the macro-scale test, the decrease of absorbance was observed both in control samples and in samples with hydrocarbons. This indicated that the organisms died. This death could have been caused by inadequate culture conditions (illumination, mixing) or by considerable fluctuations of pH (up to ±0.4 units) which was supported by literature data [5].

In the mini-scale assay with *Selenastrum capricornutum*, the changes of fluorescence caused by the compounds tested and their intermediate metabolites were determined. In the population of algae exposed to pure compounds, the chlorophyll a concentration was higher than that in control samples. Strong activity of anthracene was observed, particularly at the concentrations of $5 \cdot 10^{-7}$ mole/dm^3 (figure 3).

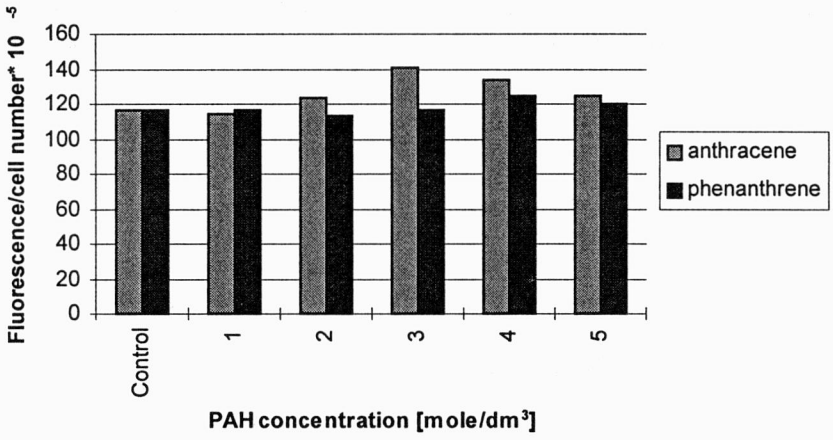

Fig. 2. Changes of fluorescence per cell number, depending on anthracene and phenanthrene concentrations in the macro-scale test; the concentrations of PAHs used: 1 – $5 \cdot 10^{-8}$, 2 – $1 \cdot 10^{-7}$, 3 – $5 \cdot 10^{-7}$, 4 – $1 \cdot 10^{-6}$, 5 – $5 \cdot 10^{-6}$ mole/dm^3

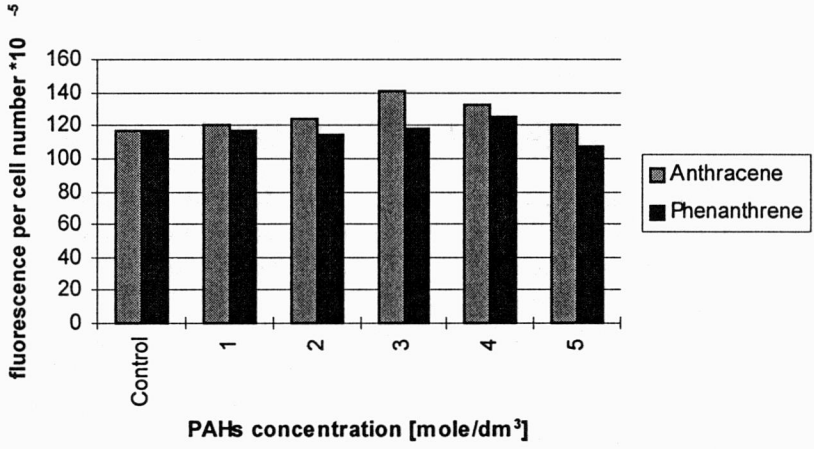

Fig. 3. Changes of fluorescence per cell number, depending on anthracene and phenanthrene concentrations in the mini-scale test; the concentrations of PAHs used: 1 – $5 \cdot 10^{-8}$, 2 – $1 \cdot 10^{-7}$, 3 – $5 \cdot 10^{-7}$, 4 – $1 \cdot 10^{-6}$, 5 – $5 \cdot 10^{-6}$ mole/dm^3

In the tests, where the activity of biodegradation products produced by two bacterial strains and the mixed culture was evaluated, significant differences of biological activity visible as the changes in chlorophyll content in algal cells were observed, depending on the compound tested. Fluorescence of the majority of samples was increased in comparison with the control sample, particularly after their exposure to biodegradation products of phenanthrene produced by the strain of *Bacillus alvei* (16A) and the mixed culture. The stimulation was 164% and 120%, respectively, in

comparison with control samples. Metabolites produced by the mixed culture, with the exception of phenanthrene derivatives, inhibited the synthesis of chlorophyll. In the case of the metabolites of the strain of *Arthrobacter* sp. (4_1'), the fluorescence was at the level of the control samples (figure 4).

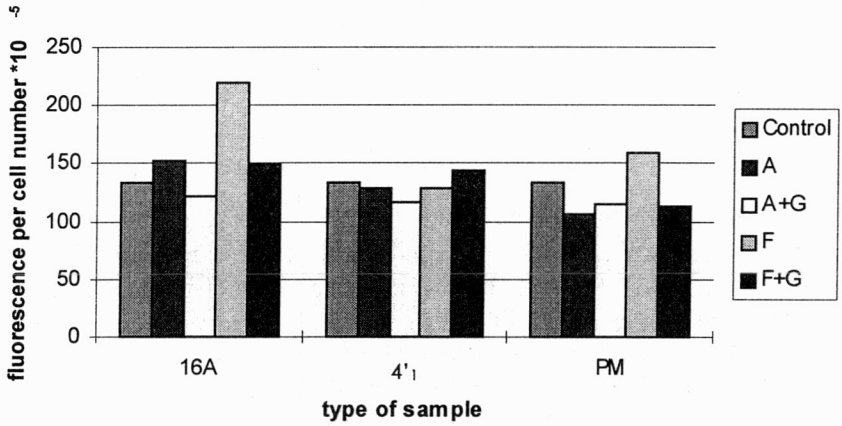

Fig. 4. Influence of biodegradation products of anthracene and phenanthrene on the changes of fluorescence per cell number in the mini-scale test; the symbols used: Control – control sample; 4_1' – metabolites produced by *Arthrobacter* sp. (4_1'); 16A – metabolites produced by *Bacillus alvei* (16A); PM – metabolites produced by the mixed bacterial culture; F+G – cometabolites of phenanthrene and glucose produced by microorganisms; F – metabolites of phenanthrene produced by microorganisms; A+G – cometabolites of anthracene and glucose produced by microorganisms; A – metabolites of anthracene produced by microorganisms

Stimulation of the number of *Selenastrum capricornutum* cells by the hydrocarbons investigated and their metabolites was found. However, SC_{20} was not established for pure hydrocarbons, while the mixture of biodegradation metabolites of PAH produced by the mixed bacterial culture ($5 \cdot 10^{-6}$ mole/dm^3) strongly stimulated cell divisions (tables 2, 3).

Table 2

Stimulating effect of anthracene and phenanthrene on the chlorophyll production by *Selenastrum capricornutum*

Concentration [mole/dm^3]	Percentage of stimulating effect [%]	
	Anthracene	Phenanthrene
$5 \cdot 10^{-8}$	109.6	104.6
$1 \cdot 10^{-7}$	102.0	104.0
$5 \cdot 10^{-7}$	102.9	114.2
$1 \cdot 10^{-6}$	102.9	101.2
$5 \cdot 10^{-6}$	114.7	109.1
Control	100.0	100.0

Table 3

Stimulating effect of biodegradation products
on the chlorophyll production by *Selenastrum capricornutum*

Type of sample	Percentage of stimulating effect [%]			
	A	A+G	F	F+G
PM	156.2	162.0	156.9	156.9
16A	108.9	103.8	103.8	106.3
4_1'	103.8	106.3	103.8	106.3
Control	100.0	100.0	100.0	100.0

Mini-scale test facilitated uniform illumination and mixing. This was reflected by the growth of algae. No changes in pH indicated that the metabolism of algae was balanced and the quality of the results was improved. The effect of pollutants was the only variable, therefore changes of fluorescence and absorbance reflected only the influence of toxic compounds. Based on these observations we conclude that mini-scale assay is a better method of measurement. It allows us to maintain stable culture conditions (mixing, illumination, pH), thus giving more reliable results than macro-scale test. Additionally, smaller sample volumes make it more cost-effective.

4. CONCLUSIONS

1. Mini-scale assay is more cost-effective with respect to sample volume and time needed to perform the test. It facilitates maintaining constant mixing and illumination conditions, uniform for all samples. Additionally, variations of pH are minimised. This influences fluorescence values and is associated with reliable results. Inappropriate pH may also alter the growth of algae.

2. The applied method of *in vivo* measurements provides reliable results (fluorescence values are correlated with chlorophyll concentration). *In vivo* measurements considerably reduce the time of the test, because the prolonged extraction procedure is eliminated.

3. Anthracene and phenanthrene are biologically active pollutants of aquatic ecosystems. Their presence in environment stimulates growth of algae and strongly influences the content of chlorophyll in their cells.

4. Biodegradation metabolites of anthracene and phenanthrene are also hazardous compounds stimulating an increase of chlorophyll a content in *Selenastrum capricornutum* cells.

REFERENCES

[1] BĄKOWSKI W., BODZEK D., *Wielopierścieniowe węglowodory aromatyczne w naturalnym środowisku człowieka – pochodzenie, występowanie, toksyczność, oszacowanie emisji w Polsce*, Archiwum ochrony środowiska, 1988, No. 3–4, pp. 197–215.

[2] REJMER P., *Podstawy ekotoksykologii*, Wydawnictwo Ekoinżyniera, Lublin, 1997.
[3] PETERSON S.A., BARICH 3rd J.J., *How Chemically Stable is Stabilized Hazardous Waste?* Remediation, Autumn, 1992, pp. 447–458.
[4] KAWECKA B., ELORANTA P.V., *Zarys ekologii glonów wód słodkich i środowisk lądowych*, PWN, Warszawa, 1994, pp. 28–30.
[5] ARENSBERG P., *A Miniscale Algal Toxicity Test*, Chemosphere, 1995, Vol. 30, No.11, pp. 2103–2115.
[6] *Standard Methods for the Examination of Water and Wastewater*, 19th Edition, 1995, pp. 8-33–8-39.
[7] PODBIELKOWSKI Z., *Glony*, Wydawnictwa Szkolne i Pedagogiczne, Warszawa, 1975, pp. 64–65.
[8] SHIROYAMA T., MILLER W.E., GREENE J.C., *Comparison of the Algal Growth Responses of Selenastrum capricornutum Printz and Anabaena flos-aquae (Lyngb.) De Brebisson in Waters Collected from Shagawa Lake, Minnesota*, Biostimulation and Nutrient Assessment, 1976, pp. 127.
[9] FITZGERALD G.P., *Factors affecting the algal procedure*, Office of Research and Monitoring U.S. Environmental Protection Agency, 1975, Washington D.C. 20460.
[10] BIŁYK A., TRACZEWSKA T.M., *Biodegradation of anthracene and phenanthrene by single strains and mixed culture of bacteria immobilised by sorption on activated carbon and zeolits*, Proceedings International Symposium Environmental Biotechnology, Oostenale, 21–24 April, 1997.
[11] TRACZEWSKA T.M., *Composition of Bacterial Mixed Culture for Biodegradation of Tricyclic PAHs in Sorption–Biodegradation System*, Proceedings International Symposium Environmental Biotechnology, Oostenale, 21–24 April, 1997, pp. 201–204.
[12] GREENE J., BARTELS C., WAREN-HICKS W.J., PETERSON S.A., *Protocols for Short-term Toxicity Screening of Hazardous Waste Sites*, EPA 600/3-88–029, 1989.
[13] *Standard Practice for Algae Growth Potential Testing with Selenastrum capricornutum*, Annual Book of ASTM Standards, Part 31 Water, 1981, pp. 1123–1128.
[14] HOFFMAN D., *Handbook of Ecotoxicology*, 1995, Lewis Publishers, London.

MOŻLIWOŚCI ZASTOSOWANIA TESTU Z *SELENASTRUM CAPRICORNUTUM* DO OCENY TOKSYCZNOŚCI MIKROZANIECZYSZCZEŃ WODY

Wielopierścieniowe węglowodory aromatyczne (WWA) należą do zanieczyszczeń wód, które w środowisku ulegają przemianom mikrobiologicznym oraz fotodegradacji, co może prowadzić do zmiany właściwości toksykodynamicznych powstałych pochodnych. Dlatego istotne było określenie wpływu antracenu i fenantrenu (jako modelowych WWA) oraz mieszaniny ich metabolitów pobiodegradacyjnych na *Selenastrum capricornutum*. Stosując test z *Selenastrum capricornutum* w skali mini i makro, określono jego przydatność do oceny stanu środowiska słodkowodnego. W miniteście, ze względu na niewielką objętość próbek, łatwiej było kontrolować i utrzymywać na stałym poziomie pH i temperaturę. Można było również efektywniej naświetlać i mieszać próbki. Uzyskane wyniki sugerują, że minitest jest wiarygodniejszy, bardziej ekonomiczny i trwa krócej niż test w skali makro.