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Chromatographic determination and toxicological potential evaluation of selected micropollutants in aquatic environment—analytical problems

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ABSTRACT

In this study, the analytical procedures for the improved extraction and determination of the selected micropollutants (anthracene, pentachlorophenol, octylphenol, benzo(a)pyrene, and diclofenac) in aqueous environment are proposed. These methods were based on application of gas chromatography coupled with mass spectrometry and solid phase extraction for isolation of tested analytes from water samples. Compared to standard procedures currently used in the range presented in this paper, the authors' modifications of analytical procedures allowed increasing the recovery rate of analytes. Within modification of procedures, hyamine, methanol were used. The substances increase solubility of analytes in water, for instance. Toxicological potential of samples containing tested micropollutants in different environmental matrix was evaluated. Proposed analytical procedures allow the quantitative determination of five different compounds in aquatic environment with satisfactory repeatability and precision of measurements. Applied modifications of analytical procedures had an influence on the increase of recovery degree of compounds. Extraction of micropollutants from effluent exceeded 60% and depended on compounds concentration in the samples, excluding the determination for lower concentration of anthracene. The limits of quantitation LOQ (in ng/L) were as follows: 6.5 for anthracene and octylphenol, 8.5 for diclofenac, and 10 for pentachlorophenol and benzo(a)pyrene. It was found that Microtox® assay allows the quick evaluation of toxicological potential of selected micropollutants. The toxicological potential (expressed as EC_{50}, in mg/L) of deionized water samples containing micropollutants was equal to: 12.8; 2.2; 1.4; 6.6; 23.1 for anthracene, pentachlorophenol, octylphenol, benzo(a)pyrene, and diclofenac, respectively. However, the toxicity was also dependent on environmental matrix. The explanation of this phenomenon requires further research.

Keywords: Micropollutants; Samples analysis; GC–MS; SPE; Microtox®

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs), endocrine disrupters compounds and pharmaceuticals are part of wide and diversified group of organic micropollutants, known as emerging pollutants, which have attracted considerable attention due to their threats to the human and aquatic organisms in recent years [1–3]. Among the different compounds of organic micropollutants, an enormous interest has been

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focused on those chemicals that are regulated by The Water Framework Directive 2000/60/EC [1,2,4,5]. Anthracene, benzo(a)pyrene, octylphenol, pentachlorophenol, and diclofenac are frequently mentioned examples of chemicals, that occur in environmental samples [1–3,6–8].

Anthracene and benzo(a)pyrene are ubiquitous environmental PAHs, mainly generated from anthropogenic processes (incomplete combustion of fossil fuels, petrochemical industry, and biomass burning). Their stability and durability are caused by their low aqueous solubility, volatility, and biodegradation. Many PAHs as well as teratogen have been defined as carcinogenic or carcinogens [9]. Octylphenol and pentachlorophenol are considered as common endocrine disrupting chemicals, that may act like hormones and induce disturbances in endocrine function—especially during early stages of the life cycle, development, pregnancy, and lactation— which cause profound and significant effects [2,10,11]. Their presence has an influence on natural bodies, even at low concentration in the environment, and can cause feminization of aquatic organisms, decrease a fertility, and survival of progeny in population [12,13]. A diclofenac is a nonsteroidal anti-inflammatory drug and has high pharmaceutical activity and, thus can have potential toxic impact on the environment [14].

Many of the micropollutants enter the conventional wastewater treatments allowing them to reach the water bodies. In this context, we need to expand our knowledge about the occurrence, fate, and toxicity of all these compounds in the environment [7,8,12,14,15].

There are many analytical methods for sensitive determination of organic micropollutants, i.e. gas chromatography or liquid chromatography coupled with mass spectrometry [6,8,16]. However, majority of them focused on surface water samples. When wastewater is in the analyzed samples, lower recovery of compounds is observed [16–20]. This is due to high complexity of the matrices in such samples. In addition, wastewater samples are complex matrices that contain large amounts of possible interfering compounds that necessitate the use of extensive extraction procedures to obtain valuable extracts to analysis.

In this study, analytical procedures for the improved extraction and determination of the selected micropollutants in aquatic samples are proposed. These methods were based on application of gas chromatography coupled with mass spectrometry and solid phase extraction (SPE) for isolation of testes analytes from water samples. The novelty of this work consists in proposed author's modifications of extraction in order to increase the recovery of analytes. Moreover, toxicological evaluation of samples containing tested micropollutants in different matrix was conducted by Microtox®.

2. Materials and methods

2.1. Apparatus, materials, and chemicals

The stock solutions of individual micropollutants (anthracene, pentachlorophenol, octylphenol, benzo(a) pyrene, and diclofenac) containing 1 μg/μL were prepared in methanol. Working standard solutions of 100 ng/μL were achieved by dilution of stock solutions. All used organic solvents were of analytical grade, purchased from Avantor Performance Materials Poland S.A. Analytical standards of micropollutants were from Sigma-Aldrich (Poznań, Poland). For extraction, SPE ENVI-18 tubes of 6 mL volume filled with 1,000 mg of phase were used, supplied by Supelco (Poznań, Poland). The extraction was carried out in SPE Cartridge Vacuum Manifold (Supelco).

2.2. Characteristics of analyzed samples

Analyses were performed with the use of two types of simulated model solutions. Those were prepared from: (1) deionized water, and (2) biologically treated wastewater by adding sufficient volume of stock solutions of micropollutants to achieve a concentration of 100, 500, 1,000, 2,000, and 5,000 μg/L.

In these studies, concentrations of micropollutants exceeded values normally observed in the environment [21]. This was done due to very small volume of extracted sample. Nowadays, increasing interest for this kind of analytical procedures are observed, because of the need to evaluate new water and wastewater treatment technologies [22–28], including, e.g. advanced oxidation processes [22,27].

The pH value of the solutions was adjusted using 0.1 M of HCl mol/L or 0.2 mol/L NaOH. In this study, biologically treated wastewater (effluent) was collected from a mechanical–biological wastewater treatment plant located in South-Eastern Poland. Effluent samples were originally free from tested compounds. The physicochemical characteristics of two types of simulated solutions are presented in Table 1.

2.3. Chromatographic analysis of micropollutants

Chromatographic analysis of micropollutants included two stages:

- Isolation of tested analytes from samples by means of SPE,

Table 1
Physicochemical characteristics of the simulated solutions

	Deionized water	Wastewater effluent
Conductivity (μS/cm)	5.180	1,058
Absorbance (UV_{254}) (1/cm)	0.000	0.218
Total organic carbon (TOC) (mg/L)	0.00	11.51

- Qualitative and quantitative analysis of extracts using gas chromatography coupled with mass spectrometry (GC–MS).

2.3.1. Extraction of anthracene and benzo(a)pyrene from water with and without modification

Anthracene and benzo(a)pyrene were separated from waters samples (20 mL) by means of SPE with the use of columns filled with octadecylsilane C_{18} bed. Before extraction, C_{18} columns beds were conditioned with methanol (5 mL) and washed by deionised water (5 mL). After the samples had completely passed, SPE bed was dried under vacuum. The extract was eluted with 3 mL of dichloromethane, and then eluate was dried under high purity nitrogen flux. Dried residue was re-dissolved in 50 μL of methanol and analyzed using gas chromatography. This extraction was performed according to method described elsewhere [29,30]. Modification of analytical procedure included: (1) adding to the water samples an aqueous solution of 0.004 mol/L hyamine reagent (5 mL/L) supplied by Merck (Warsaw, Poland) before extraction, and (2) adding 50 μL of methanol to the top of C_{18} bed before elution.

2.3.2. Extraction of pentachlorophenol, octylphenol, and diclofenac with and without modification

For separation of pentachlorophenol, octylphenol, and diclofenac was also used SPE with the same columns as in the point 2.3.1. However, there were some differences in extraction conditions. Firstly, C_{18} beds were conditioned with acetonitrile (5 mL), methanol (5 mL), and washed by deionized water (5 mL). The volume of samples which passed through SPE cartridges was 20 mL. Secondly, the analytes were eluted with 3 mL of acetonitrile/methanol (60/40, v/v). After evaporation of solvent from eluate under a gentle N_2 flow, analytes were dissolved in 50 μL of methanol and analyzed with GC–MS. Extraction procedure was similar to published elsewhere [31]. Modification of analytical procedure included the addition of methanol (5 mL/L) to water samples before extraction.

2.3.3. GC–MS analysis

The quantitative analysis was made with the use of Saturn 2100 T gas chromatograph (GC) coupled with mass spectrometer (MS) of ion trap type with electron ionization (EI) by Varian (Warsaw, Poland). Chromatograph was equipped with 30 m × 0.25 mm i.d. VF-5 ms capillary column of 0.25 μm film thickness by Varian. Helium 5.0 was used as the carrier gas. The temperature of ion trap and ion source was 200°C.

For quantitative analysis, MS was operated in selected ion monitoring (SIM) mode. The quantitative calculations were carried out on the basis of measurements of peak area, which were then compared with data of standard solutions. The GC–MS conditions described above were identical for all determined micropollutants. The observed variations are connected with temperature of program and injector (Table 2). Chromatographic separation of micropollutants was performed by two different temperature program of column oven, i.e. the range of 50–260°C for anthracene and benzo(a)pyrene and 80–220°C for pentachlorophenol, octylphenol, and diclofenac. The injector temperature of first program was set at 240°C and for the second one was equal to 230°C.

Table 2
GC–MS conditions for micropollutants analysis

	Compound	
Parameter	Anthracene, benzo(a)pyrene	Pentachlorophenol, octylphenol, diclofenac
Carrier gas flow rate[a] (mL/min)		1.1
Injected volume (μL)		1, 2 or 3
Injector temperature (°C)	240	230
Oven program	50°C (4 min)-8°C/min→260°C (15 min)	80°C (8 min)-4°C/min→220°C (5 min)

[a]Helium (5.0).

Table 3
Samples toxicity classification system [31–34]

Effect (%)	Toxicity class
<25	Non toxic
25–50	Low toxic
50.1–75	Toxic
75.1–100	Highly toxic

2.3.4. Toxicological evaluation

Toxicological evaluation was carried out by means of commercial assay system Microtox® using the bioluminescent photobacterium *Vibrio fischeri*. The exposure of the bacteria to toxic substances leads to changes in the metabolic processes, which simultaneously causes changes in the intensity of light emitted by the micro-organisms [32]. The tests were performed using the Microtox Omni system in the Microtox 500 analyser by Tigret Ltd. (Warsaw, Poland) serving as both an incubator and a photometer. Toxicity evaluation was performed by the analysis of the simulated solutions of different micropollutants concentrations (100 μg/L, 500 μg/L, 1,000 μg/L, 2000 μg/L, and 5,000 μg/L). Then, suspension of rehydrated bacteria was added to the water samples. After 5 min of exposure, percent bioluminescence inhibition was measured against the control sample (2% NaCl).

The toxicity of the samples was classified using a straightforward system used by many researchers [32–35], which is based on the magnitude of the observed effect induced in the indicator organisms (Table 3).

3. Results and discussion

3.1. Detection limit and precision

The analyses of tested compounds were based on SIM method, listed in Table 4. In order to increase sensitivity in GC–MS analysis, more than two ions were used for identification of tested compounds.

The comparison of retention time plays an important role in qualitative analysis. In our case, the identification based on the comparison of retention time was very accurate. Determined retention times from many repetitions of chromatographic isolations differ slightly, as evidenced by low value of standard deviation (SD) of this parameter in each case as well as high measurement precision—expressed by low values of variation coefficient (CV); from 1.95 to 4.99 (Table 4).

The limits of detection were calculated as the concentration (in ng/L) with a signal-to-noise (S/N) ratio above three and was equal to 0.3 for anthracene, octylphenol, and diclofenac and 0.6 for pentachlorophenol and benzo(a)pyrene.

Repeatability of quantitative analysis is fundamental for determining the concentration of micropollutants in environmental samples. For several (selected) concentration levels that were injected onto chromatographic column, the precision of mass detector response was calculated and presented in Table 5. Coefficient of variation (CV, %) of these measurements was in the range from 2 to 8%, which indicates the good precision.

3.2. Recovery studies

In the next stage of study, repeatedly prepared deionized water solutions with micropollutants containing 100 and 500 μg/L were extracted according to procedures with and without modification (as described in point 2.3.1 and 2.3.2). Based on obtained chromatographic data, recovery of each determined micropollutants was calculated (Table 6). These results are graphically presented in Fig. 1. Average values of this parameter in all cases were higher for the procedure with modification and ranged from 17 to 96% and from 24 to 100% for the concentration of the compounds at the level of 100 and 500 mg/L, respectively.

Table 4
Quantitative analysis parameters

Compound	Selected ions in SIM (m/z)	Retention time, $t_R \pm$ SD ($n = 5$)	CV (%) ($n = 5$)	Limit of detection LOD[a] (ng/μL)
Anthracene	87; 126; 152; 178	23.590 ± 0.017	4.99	0.3
Pentachlorophenol	95; 130; 165; 202; 230; 266	32.730 ± 0.010	3.24	0.6
Octylphenol	107; 149; 171; 206	33.281 ± 0.017	2.97	0.3
Benzo(a)pyrene	126; 149; 174; 200; 224; 252	38.559 ± 0.011	3.34	0.6
Diclofenac	151; 179; 214; 242; 277	42.871 ± 0.020	1.95	0.3

[a]Signal to noise ratio (S/N)>3; SD—standard deviation; n—number of analysis; CV—coefficient of variation.

Table 5
Precision of mass detector response

	Concentration (ng/μL)			
	CV (%) ($n = 5$)			
Compound	50	30	20	10
Anthracene	5	4	5	8
Pentachlorophenol	3	3	5	2
Octylphenol	3	2	3	3
Benzo(a)pyrene	3	5	3	6
Diclofenac	2	3	3	3

Notes: CV—coefficient of variation; n—number of analysis.

The use of an aqueous solution of hyamine reagent or methanol in modified analytical procedure resulted in higher solubility of micropollutants in samples, and thus the performance of extraction was higher [36,37]. Moreover, under these conditions, adsorption of analytes on laboratory glassware is limited.

The recovery of each micropollutants from wastewater was also evaluated. In the present case, however, only modified extraction procedure was used. The efficiency of extraction of micropollutants exceeded 60 and 71% for concentration of compounds in water at the level of 100 and 500 μg/L, respectively. Excluding the determination of lower concentration of

Table 6
The efficiency of micropollutants extraction and accuracy of the SPE-GC/MS procedure for wastewater effluent

	Concentration				
	100 μg/L		500 μg/L		
Compound	Recovery (%) ($n = 5$)	SD (%)	Recovery (%) ($n = 5$)	SD (%)	LOQ[a] (ng/L)
Anthracene	28	1	71	6	6.5
Pentachlorophenol	82	1	99	3	10.0
Octylphenol	78	2	99	1	6.5
Benzo(a)pyrene	62	3	98	1	10.0
Diclofenac	60	2	79	1	8.5

[a]Signal to noise ratio (S/N)>10; SD—standard deviation; n—number of analysis.

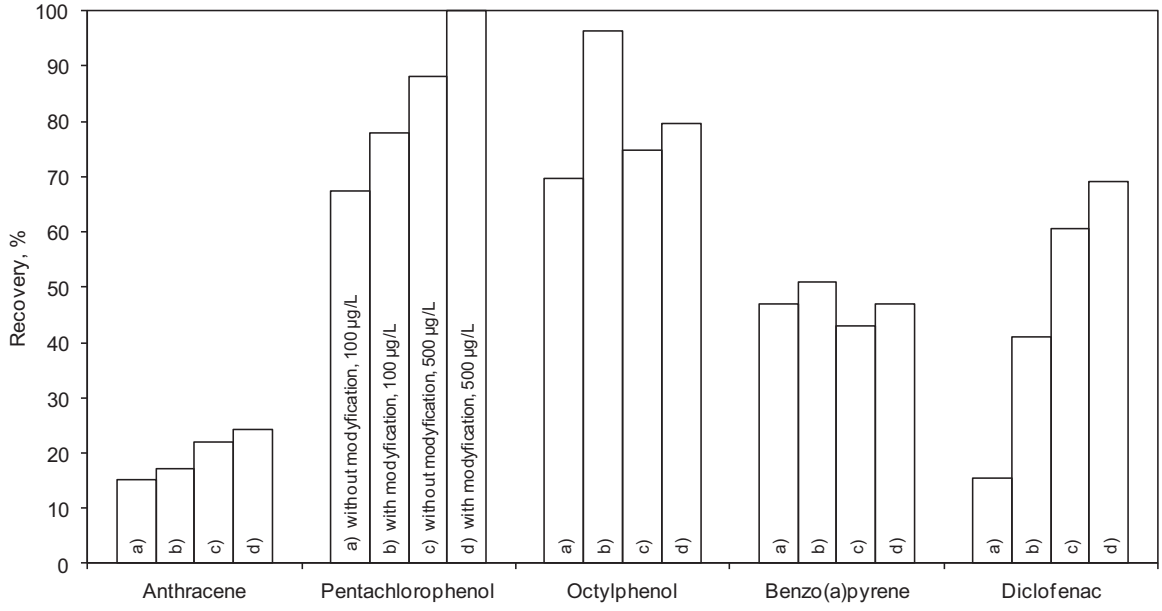

Fig. 1. Micropollutants recovery (n, number of analysis – 4) in analytical procedure without and with modification (compounds concentration 100 μg/L and 500 μg/L in deionized water).

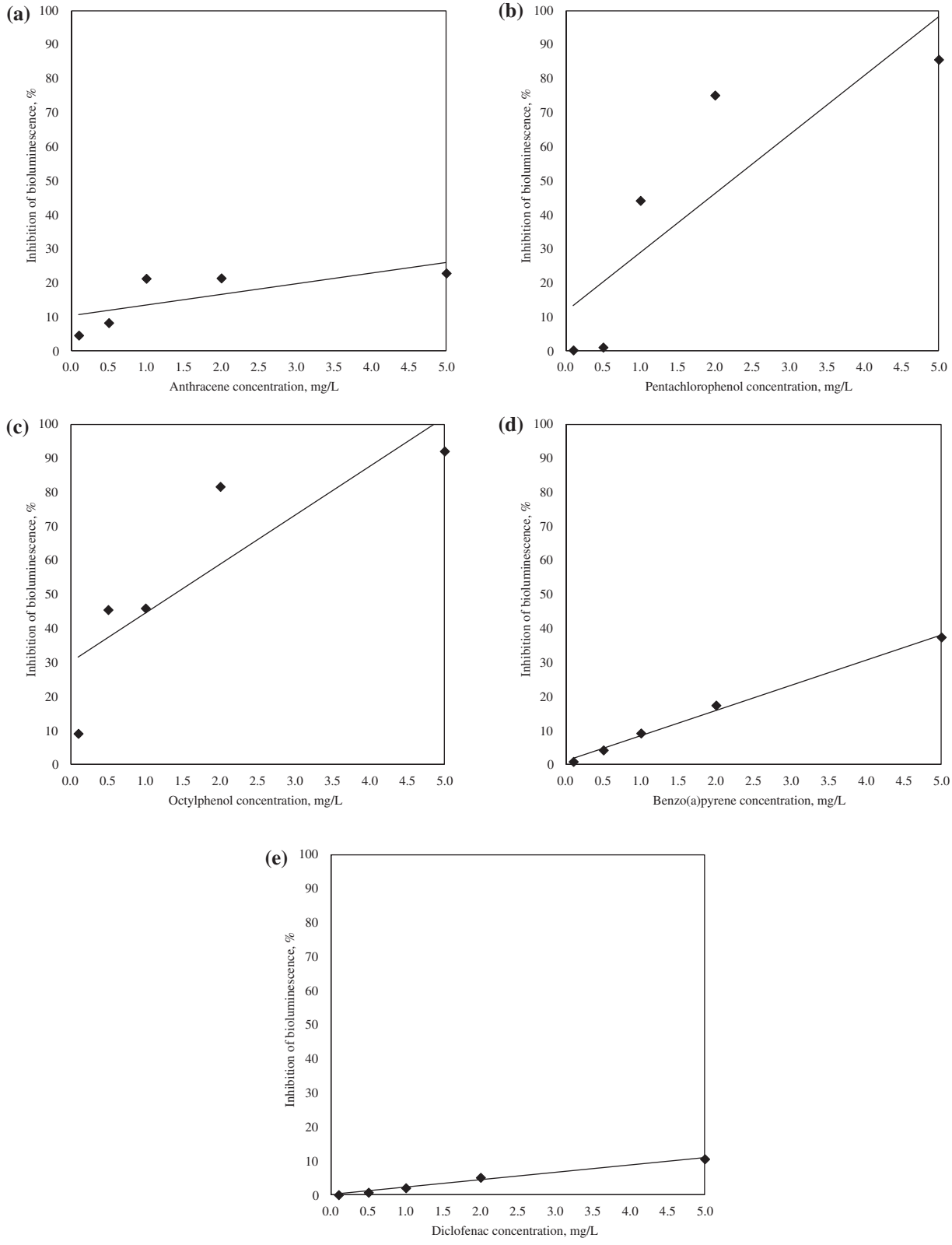

Fig. 2. Levels of bioluminescence inhibition for anthracene (a) pentachlorophenol (b) octylphenol (c) benzo(a)pyrene (d) diclofenac and (e) solutions (exposure time: 5 min).

Table 7
EC_{50} values computed based on the toxicity test results

Compound	EC_{50} (mg/L)
Anthracene	12.8
Pentachlorophenol	2.2
Octylphenol	1.4
Benzo(a)pyrene	6.6
Diclofenac	23.1

anthracene, the difference in results achieved for individual series did not differ more than 6%. In this case, limits of quantitation (ng/L) were 6.5 for anthracene and octylphenol, 8.5 for diclofenac, and 10 for pentachlorophenol and benzo(a)pyrene. The limits of quantitation were calculated as the concentration (in ng/L) with S/N ratio above 10. The recovery of micropollutants from wastewater was slightly higher than from deionized water. It could be attributed to the occurrence of some inorganic substances in wastewater, that enhance extraction (salinity effect) [18,38].

3.3. Toxicity analysis

The dependence between toxic effect of deionized water samples (containing separate tested micropollutants) and the concentration of compounds was measured using the Microtox® toxicity assay (Fig. 2). Due to the fact, that low toxicity was observed during studies involving extraction performance of samples containing 100 and 500 μg/L of micropollutants, the toxicity assays were also performed for solution of higher compounds concentration, i.e. 1,000, 2,000 and 5,000 μg/L.

It was found that the bioluminescence inhibition value increased with increasing micropollutants concentration in water samples.

In similar experiment, however with biologically treated wastewater, similar trend was observed. Although, toxic effect was on average 10% lower in comparison to the results for deionized water. The explanation of this phenomenon requires a further research. The bioluminescence inhibition depends both on the type of compound and tested water matrix.

The interpretation of obtained toxicological data has been carried out on the basis of samples toxicity classification system (Table 3). It was found, that the solutions containing pentachlorophenol (Fig. 2(b)) and octylphenol (Fig. 2(c)) in concentration exceeding 2 mg/L indicated bioluminescence inhibition at the level higher than 75%; corresponding to the high toxicity.

Observed low bioluminescence inhibition percentage induced by anthracene (Fig. 2(a)), benzo(a)pyrene (Fig. 2(d)), and diclofenac (Fig. 2(e)) indicated that these samples were not toxic, excluding the benzo(a) pyrene solution of 5 mg/L.

Moreover, we evaluated toxicological potential of tested micropollutants expressed by EC_{50}, calculated in mg/L (Table 7). The EC_{50} value (mg/L) amounted to 12.8, 6.6, and 23.1 for anthracene, benzo(a)pyrene, and diclofenac, respectively, and thus exceed concentration of compounds tested in this study. It should be emphasized that for both tested PAHs, the EC_{50} values are higher than their solubility in water (anthracene 0.044 mg/L and benzo(a)pyrene 0.00147 mg/L) [39]. It might be a result of higher solubility of these compounds, enhanced by preparation of standard solution in methanol (which added to simulated water samples). The EC_{50} values (in mg/L) were 2.0 and 1.4 for pentachlorophenol and octylphenol, respectively.

4. Conclusions

- Two presented analytical procedures using SPE extraction techniques and qualitative–quantitative analysis GC–MS allow quantitative determination of five different micropollutants in water environment with satisfying repeatability and measurement accuracy. Applied modifications of analytical procedures had an influence on the increase of compounds recovery. Determined recoveries allow full control of quantitative determination of tested compounds. Micropollutants extraction capacity exceeded 60% for compound concentration in water equal to 100 μg/L and 71% for concentration of 500 μg/L, excluding determination for lower concentration of anthracene. Limits of quantitation (in ng/L) were as follows: 6.5 for anthracene and octylphenol, 8.5 for diclofenac, and 10 for pentachlorophenol and benzo(a)pyrene.
- Appliance of Microtox® assay allow the determination of toxicological potential of selected micropollutants in relatively short time. For deionized water solution, toxicological potential of particular micropollutants (expressed as EC_{50} in mg/L) was: 12.8 (anthracene), 2.2 (pentachlorophenol), 1.4 (octylphenol), 6.6 for benzo(a)pyrene, and 23.1 (diclofenac). However, results of the test were dependent on type of environmental matrix. Explanation of this phenomenon requires conducting further research in this matter.

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