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GRZEGORZ NAŁĘCZ-JAWECKI[1], STANISŁAW BARAN[2], JOANNA MANKIEWICZ-BOCZEK[3], ELŻBIETA NIEMIRYCZ[4], LIDIA WOLSKA[5], JOANNA KNAPIK[6], KATARZYNA PIEKARSKA[7], MARIA BARTOSIEWICZ[8], GRZEGORZ PIĘTOWSKI[9]

THE FIRST POLISH INTERLABORATORY COMPARISON OF THE LUMINESCENT BACTERIA BIOASSAY WITH THREE STANDARD TOXICANTS

Quality assurance test with standard toxicants should be carried out regularly to check the sensitivity of the testbiont and the quality of the procedure. In the luminescent bacteria bioassay, zinc sulphate and phenol have been used as the respective inorganic and organic reference substances. ISO 11348 standard proposes 3,5-dichlorophenol (DCP) as a standard toxicant for luminescent bacteria toxicity assay. This work presents the results of the first ring study performed by 9 Polish laboratories. One hundred and twenty five valid toxicity data were received and only 7.2% data were rejected. In the case of DCP, all results were valid and the coefficient of variation for this compound was the lowest.

[1] Department of Environmental Health Sciences, Medical University of Warsaw, ul. Banacha 1, 02-097 Warsaw, Poland. E-mail: grzegorz.nalecz-jawecki@wum.edu.pl

[2] Institute of Soil Science and Environment, University of Life Sciences in Lublin, ul. Leszczyńskiego 7, 20-069 Lublin, Poland.

[3] International Institute PAS European Regional Centre for Ecohydrology u/a UNESCO, ul. Tylna 3, 90-364 Łódź, Poland.

[4] Institute of Oceanography, Departament of Marine Chemistry and Environmental Protection, Laboratory of Persistent Organic Pollutants, University of Gdańsk, al. Marszałka Piłsudskiego 46, 81-378 Gdynia, Poland.

[5] Chemical Faculty, Gdansk University of Technology, ul. Narutowicza 11/12, 80-952 Gdańsk, Poland.

[6] Water Supply and Sewage Operator Company Ltd. in Boleslawiec, ul. Lasicka 17, 59-700 Bolesławiec, Poland.

[7] Institute of Environment Protection Engineering, Wrocław University of Technology, ul. Wybrzeże Wyspiańskiego 27, 50-370 Wrocław, Poland.

[8] Departament of Environmental Protection, Medical University of Gdańsk, ul. Powstania Styczniowego 9B, 81-519 Gdynia, Poland.

[9] TIGRET Ltd., ul, Warszawska 27, 02-495 Warsaw, Poland.

1. INTRODUCTION

Poland is numbered among European countries with poor water resources [1]. Since 1990 there has been a downward trend in water abstraction. In the time span of 1990–2005, the total annual water abstraction per capita decreased by 20% [1], [2]. Bacteria have been widely used for the valuation of toxicity [3] and mutagenicity [4] of drinking water resources.

Vibrio fischeri previously known as *Photobacterium phosphoreum* has been used in ecotoxicology for 30 years [5]. The luminescent bacteria toxicity bioassay developed in the late 70's has been available in the commercial version as the Microtox® assay since 1980. Later, other commercial systems were brought onto the market, e.g. ToxAlert (Merck, Darmstadt, Germany), BioFixLumi (Macherey-Nagel, Duren, Germany), Tox-Tracer (Skalar, Breda, the Netherlands) and LUMIStox (Hach-Lange, Düsseldorf, Germany), DeltaTox (SDI, Newark DE, USA). The systems use non-pathogenic luminescent bacteria, *V. fischeri*, which emit light as a result of their metabolism. The bacteria are incubated with the sample for a very short time of 5–30 min. The reduction in light intensity provides a measure of the toxicity of the sample. The main advantages of the systems are the speed and simple operational procedure. The bacteria are sold in a lyophilised form, thus no culture is needed and the results from different laboratories are comparable [6]. They are one of the most often used bioassays applied in testing pure compounds [7] and environmental samples [8]. Microtox® was also applied in the evaluation of the efficacy of photodegradation process [9].

The first Polish Microtox® device was purchased by the Department of Environmental Health Sciences of the Medical University of Warsaw, 15 years ago and it is still in use [10]. Now more than 10 systems are used in Poland, in different institutes, both scientific and commercial ones.

The luminescent bacteria toxicity bioassay is used for testing new chemical compounds, and the existing toxicity databases comprise data for several hundreds of chemicals [11]. However, the main application of the test is monitoring industrial effluents to control their quality and to detect unwanted toxicity in treatment stations.

The quality assurance test with standard toxicants should be carried out regularly to check the sensitivity of the testbiont and a quality of the procedure. In the Microtox® system, zinc sulphate and phenol have been the inorganic and organic reference substances since the early days of commercialisation of the assay. ISO 11348 standard [12] proposes 3,5-dichlorophenol as a standard toxicant for luminescent bacteria toxicity assay. Its toxicity has been found very consistent [13].

This work presents the results of the first ring study performed by 9 Polish laboratories. Three toxicants were tested in different commercial devices based on luminescence inhibition assay with *V. fischeri*. Reproducibility was based on the evaluation of the median effective concentration that gives 50% of inhibition EC50.

2. MATERIALS AND METHODS

2.1. STANDARD SAMPLES

Three reference toxicants were selected in the interlaboratory study. Solutions of phenol (BDH), 3,5-dichlorophenol (Fluka) and zinc sulphate ($ZnSO_4 \cdot 7H_2O$, POCh) were prepared in the deionised water at concentrations of 75 mg dm^{-3}, 20 mg $dm^{-3}$and 15 mg dm^{-3}, respectively. The stock solutions were prepared by the central laboratory, then distributed in polyethylene containers among the participant laboratories. Samples were stored at 4–8 °C and analysed within a month.

2.2. THE LUMINESCENT BACTERIA TOXICITY BIOASSAY

The participant laboratories used reagent (*V. fischeri* NRRL-B 11177) from SDI (*via* Polish distributor, Tigret). The reconstitution of the reagent was carried out according to the standard operational protocol. Samples were analysed mainly in the Microtox® M500 analysers; however, two DeltaTox® and ToxAlert® devices were also applied. Dilution of the samples was performed using 2% NaCl solution. The percentage of the inhibition of the luminescence was determined after 5 and 15 min of exposition. On this basis the 5min-EC50 and 15min-EC50 were calculated. When the sample was analysed with the MicrotoxOmni® software, 95% confidence range and confidence factor were noted. Each sample was analysed by each laboratory three times. The confidence factor (CF) is the ratio of the upper level of the 95% confidence range to the EC50 values.

2.3. STATISTICAL TREATMENT OF THE RESULTS

The mean, standard deviation of the mean (SD) and median were calculated for each toxicant. The outliers were rejected with the use of Dixon *Q*-test for 95% confidence level. Quality indicator *z*-score was calculated according to the formula:

$$z\text{-score} = (X_n - \text{mean})/\text{SD}, \tag{1}$$

where X_n is the result.

The *z*-score classification was based on HERNANDO's et al. [14] data: (satisfactory $|z| \leq 2$, questionable $2 < |z| \leq 3$, unsatisfactory $|z| > 3$).

3. RESULTS AND DISCUSSION

Nine laboratories participated in the first Polish interlaboratory comparison. There were over 162 EC50 results to be expected. These results correspond to the determination of the toxicity of the same 3 samples in 3 runs with 2 incubation times by 9 par-

ticipants. However, for zinc 5min-EC50 the results were not taken into consideration. Additionally, DeltaTox® instrument enables the measurement of only a single incubation. Thus, 125 valid toxicity data were received (table 1). Nine results were rejected, 3 of them were not quantitative values, the toxicity was lower than the highest concentration tested. Six data were unreasonably different from the others and were rejected on the basis of the Dixon *Q*-test.

Table 1

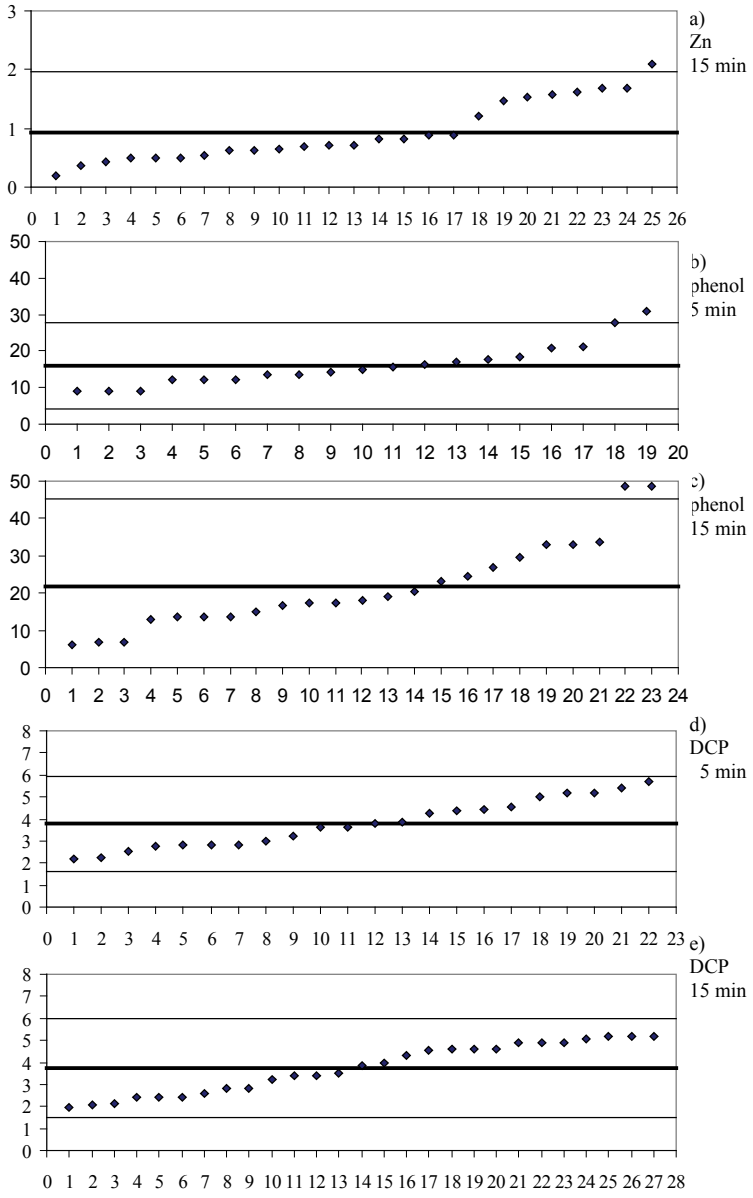
Number of results and variation coefficients in interlaboratory comparison

	Number of results		CV (%)	CF > 2	$\|z\| > 2$
	All	Rejected			
Zn 15 min	28	3	55	11	1
DCP 5 min	22	–	29	1	–
DCP 15 min	27	–	30	–	–
Phenol 5 min	21	2	37	–	1
Phenol 15 min	27	4	55	3	2
	125	9	–	15	4

CV – coefficient of variation
CF – coefficient factor, only for results calculated with MicrotoxOmni® (19 results)

The coefficient of variation (CV) was high ranging from 29% for DCP to 55% for zinc and phenol (table 1). The results are comparable to those received in the first European comparison organised in 1993 in Spain [15]. The CV was high – 34.7% and 41.2% for phenol and zinc, respectively, though the participants were using bacteria from the same lot number and they were using a standardised experimental procedure – Microtox® Basic protocol. However, according to the coordinator [6] some of the participants had only short-term experience with the Microtox® test. During the next intercalibrations organised by the same group of Spanish scientists the CV slowly decreased [6]. In 2005, the average CV was 27% ranging from 15% to 33% in the case of DCP and zinc ions [13]. According to literature sources, toxicity of metals shows a CV much higher than that of organics, especially in the case of not ionised and not volatile organics such as DCP [11], [13].

Coefficient factor (CF) is calculated by the MicrotoxOmni® software. It is the ratio of the 95% upper confidence level to the EC50 value. It shows the dispersion of the toxicity results of the dilution series of an individual sample. In our data set, the CF value was higher than 2 only once in the of case of DCP and 11 times for zinc. It clearly indicates that for DCP the toxicity was proportional to the concentration of the compound, while in the case of zinc the correlation between the toxicity and the concentration was not good, which might influence the EC50 values.



Display of EC50 values for all samples

The mean EC50 values received in our study fall within reported literature values for these compounds (table 2). The figure displays all of the EC50 values obtained for 3 toxicants with marked mean values, upper and lower limits calculated as ± two *z*-score values.

Table 2

The values of EC50 for standard toxicants used in the comparison

	Zn^{2+} 15 min-EC50 (mg dm^{-3})	Phenol 5min-EC50 (mg dm^{-3})	3,5-dichlorophenol 15min-EC50 (mg dm^{-3})
Range according to SDI	0.6 – 2.2	13.0 – 26.0	–
	1.4 – 8. 0 [1]	21.1 – 35.8 [2]	2.77 – 8.17 [2]
	1.45 (0.58 – 2.56) [3]	–	3.47 (2.02 – 4.15) [3]
This intercalibration	0.93 (0.20 –2.10)	16.0 (9.0 – 30.7)	3.74 (1.98 – 5.20)

[1] – MUNKITTRICK et al. [17]
[2] – KAISER and PALABRICA [11]
[3] – RIBO and RIVA [13]

Phenol has been the organic reference substance proposed by the supplier of the luminescence bacteria since the commercialisation of the assay. It is an acute toxic organic which exhibits toxicity even after 5 min of exposure. In our comparison, the toxicity of phenol after 15 min was slightly lower than after 5 min (table 3). This may be caused by the higher dispersion of the 15 min-EC50 data. In the case of phenol, 6 outliers were rejected and additional 3 data were questionable with *z*-score higher than 2. The high dispersion of the results may be due to the lower toxicity of the sample containing phenol in comparison with the other tested samples. Higher variability in samples with low toxicity may be caused by the susceptibility limit of the bacteria and the detection limit of the luminometer [6].

Table 3

Statistical parameters for standard toxicants used in the comparison

	Zn	Phenol		DCP	
	15 min	5 min	15 min	5 min	15 min
Number	25	19	23	22	27
Mean	0.93	16.0	21.7	3.78	3.74
SD	0.51	5.90	11.8	1.09	1.13
CV	55%	37%	55%	29%	30%
Median	0.72	15.0	18.0	3.70	3.87
Maximum	2.10	30.7	48.7	5.71	5.20
Minimum	0.20	9.0	6.0	2.20	1.98
Mean / median	1.3	1.1	1.2	1.0	1.0

3,5-DCP is an organic substance 5-fold more toxic than phenol. In our comparison, the 5 min-EC50 and 15 min-EC50 values were almost identical. The dispersion of the DCP results was low (figure) and no outliers and results with *z*-score > 2 were noted (table 1). Additionally, median values are very close to the mean values indicating the even distribution of the data.

Zinc sulphate is the inorganic reference substance used in quality assurance tests of luminescence bacteria. It was applied in all previous intercalibrations [6], [16]. Its toxicity strongly depended on the time of incubation, it was low after 5 min of incubation and rapidly increased after 15 and 30 minutes. According to the published reports [17] the highest concentration of zinc sulphate used in our study should not had been toxic to the *V. fischeri* after 5 min of incubation. However, two out of nine participants reported the 5-min values. It may be caused by long measurement time of individual dilutions of the sample. In the statistical calculations 5-min EC50 values were not taken into consideration. The toxicity of zinc also strongly depends on the pH of the sample. As the 2% NaCl used as a diluent is not buffered, variations of the pH of zinc solutions may be observed causing the variations of toxicity. RIBO and RIVA [13] applied saline buffered according to ISO [12] in their intercalibration. They observed lower toxicity of zinc, but also lower coefficient of variation.

4. CONCLUSIONS

The results received in the first Polish interlaboratory comparison of the luminescent bacteria bioassay were comparable to the previous European intercalibrations.

The coefficient of toxicity variation result received for 3,5-dichlorophenol is the lowest comparing to that for the zinc ions and phenol.

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