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APPLICATION OF BIOTESTS IN CYANOBACTERIAL EXTRACT TOXICITY ASSESSMENT

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Abstract: The aim of the study was to determine the toxicity of the extract obtained from the cyanobacterial cells derived from the waters of Zemborzycki dam reservoir with use of a battery of biotests. The taxonomic identification of the bloom-forming cyanobacteria revealed high abundance of *Aphanizomenon flos-aquae* and *Dolichospermum* spp. (*Anabaena* spp.) and in a lower degree of *Microcystis aeruginosa* and *Planktothrix agardhii.* In the extract obtained from concentrated cyanobacterial cells, hepatotoxin microcystin-LR at a concentration of 22.89 ± 3.74 µg/L and neurotoxin Antx-a at 13.02 ± 0.01 µg/L have been detected. Toxicity of the extract was evaluated with the following assays: Daphtoxkit F magna with the crustacean *Daphnia magna*, Thamnotoxkit F with the crustacean *Thamnocephalus platyurus*, Rotoxkit F with the rotifer *Brachionus calyciflorus* and Protoxkit F with ciliate *Tetrahymena thermophila*. The most sensitive organism among all studied was *T. platyurus* for which EC_{50} was estimated to be 1.2% of the initial extract concentration. On the basis of the highest obtained value of the toxicity unit (TU = 83) the studied sample was classified to the IV class, which is of high acute toxicity. Additionally, it was found that reactivity on cyanobacterial products differs greatly among organisms used in bioassays, which indicate the need for using a set of biotests.

INTRODUCTION

Cyanobacterial water blooms, appearing in many fish ponds and recreational reservoirs in the Lubelszczyzna region, are one of more significant factors responsible for water quality deterioration [6–8]. The water blooming phenomenon involves a range of negative effects, causing such changes of abiotic conditions, as dissolved oxygen decrease, the pH change, the increase of water turbidity and weaker sunlight permeability, leading to changes in the whole aquatic ecosystem [9].

Another factor that should be taken into account when assessing the negative effects of blooms is the ability of cyanobacteria to produce and release cyanotoxins. These substances are a class of diverse chemical compounds of multi-mode biological activities. On the basis of their structure, they are divided into cyclic peptides, alkaloids, and lipopolysaccharides (LPS), and when their direct toxic effects are considered, to hepatotoxins, neurotoxins, cytotoxins and dermatotoxins [5, 12]. Most of them are

secondary metabolites, except of LPS, an integral component forming the outer membrane of cyanobacteria. Cyanotoxins are released directly into water as a result of cell lysis during cyanobacterial bloom decay, which can lead to the local occurrence of very high concentrations of these substances [5, 6].

So far, in Lublin province water bodies, hepatotoxins – microcystins (MCs), with the most toxic variant, MC-LR, and a potent neurotoxin – anatoxin-a (Antx-a) have been identified [6–8, 11].

Zemborzycki dam reservoir is formed on the Bystrzyca River, situated within the administrative boundaries of the city of Lublin. Currently, the reservoir plays mainly recreational functions for the residents of Lublin and it is frequently visited by anglers. Zemborzycki reservoir, similarly to many other dam reservoirs [7, 9, 12], is highly eutrophicated. This contributes to frequently observed water blooms caused by toxinogenic cyanobacteria [8].

The aim of this study was to evaluate the toxicity of the extract obtained from cyanobacterial cells obtained from the Zemborzycki dam reservoir during water blooming, with the use of the battery of bioassays.

MATERIAL AND METHODS

The water sample was taken under calm weather conditions from the surface layer (0.5 m) from the Zemborzycki reservoir during massive cyanobacterial water bloom in June 2011. The sampling point was situated on the west side of the reservoir, near the beach. The surface water (50 L) was filtered with the plankton net and the cyanobacterial cells were concentrated to the final volume of 1.5 L.

Taxonomic identification of cyanobacteria

Qualitative and quantitative analyses of the obtained cyanobacteria were performed under a light microscope using a Bürker chamber. Results are given as a number of cyanobacteria individuals (bundles of *Aphanizomenon flos-aquae*, trichomes of *Planktothrix agardhii*, bends of *Dolichospermum* spp. and colonies of *Microcystis aeruginosa*) per 1 L of the concentrated sample.

Extract preparation

The extract from the concentrated cyanobacterial cells was obtained for further cyanotoxin analysis and the toxicity assessment. The sample was ultrasonically disintegrated in an ice bath with a disintegrator Omniruptor 400 (3 × 10 min, 70% of power). Then it was centrifuged at 17000 × *g* for 10 min at 10°C (MPW 351R), the supernatant was decanted, filtered with 0.45 µm filters (Millipore) and immediately frozen at -40°C for further determinations.

Cyanotoxin determination

Antx-a determination in the obtained extract was performed by the high pressure liquid chromatography (HPLC) with a fluorescence detector (Shimadzu), after fluorimetric derivatisation with 4-fluoro-7-nitrobenzofurazan (NBD-F, Fluka), using the Kinetex C-18 column (2.6 µm; 100 mm × 3.0 mm). Toxin detection was conducted according to a method described by James et al. [2]. In brief, the mobile phase was a mixture of acetonitrile (Merck)/water (45:55, vol/vol) supplemented with 0.05% trifluoroacetic acid

(TFA, Merck). Detection was performed at Ex = 470 nm, Em = 530 nm, with a flow rate of 0.5 ml/min. As a standard for the calibration curve preparation, Antx-a fumarate (Tocris, Bioscience) was used.

Determination of MC-LR was performed using HPLC with diode array detector (Shimadzu). The sample volume of 10 µl was dosed on Kinetex C-18 column. The mobile phase consisted with acetonitrile and water with 0.05% TFA, with a gradient run and a flow rate at 0.5 ml/min. Detection was performed at a wavelength λ = 238 nm, with MC-LR (Alexis Biochemicals) used as a standard for a quantitative estimation of the toxin concentration.

Extract toxicity determination

The toxicity of the obtained extract was evaluated with the use of the battery of biotests consisted with the following assays: Daphtoxkit F magna (*Daphnia magna*), Thamnotoxkit F (*Thamnocephalus platyurus*), Rotoxkit F (*Brachionus calyciflorus*) and Protoxkit F (*Tetrahymena thermophila*) (MicroBioTests Inc., Belgium). All determinations were performed strictly according to producer protocols, compatible with OECD Guideline 202 standards, ISO 14380 and ASTM E1440-91, respectively. Each assay was performed twice.

Toxicity data analysis

Toxicity data obtained from the bioassays were expressed as the percentage of toxic effects (PE) in the comparison to the control. The extract dilution was treated as non toxic, if PE $\leq$ 20%. EC_{50} (50% effective concentration) values, understood as the extract dilutions causing death or any visible disturbances of the normal activity of the half of the exposed population, were calculated using EPA Probit Analysis Program, Ver. 1.5. In the case of IC_{50} (50% inhibitory concentration), that is the extract dilution causing an appropriate growth inhibition of the exposed population (calculated in Protoxkit F), the value was estimated with the use of spreadsheet software provided by the assay producer. Finally, the obtained EC_{50} and IC_{50} values were converted into toxic units (TU) with the formula: $TU = (1/ EC_{50}) \cdot 100$ and the class of toxicity of the studied sample was determined according to Persoone et al. [10].

RESULTS AND DISCUSSION

The concentrated sample with cyanobacteria contained mainly *Aphanizomenon flos-aquae* (111×10^7/individuals L) and *Dolichospermum* spp. (*Anabaena* spp.) (513×10^7/individuals L). In a lower abundance *Microcystis aeruginosa* (1.34×10^7/individuals L) and *Planktothrix agardhii* (0.33×10^7/individuals L) were present. All of the cyanobacterial species found in the sample are potential producers of cyanotoxins.

The toxin presence in the obtained extract was confirmed in further analysis, in which MC-LR at a concentration of 22.89 ± 3.74 µg/L and Antx-a at 13.02 ± 0.01 µg/L were detected. These two toxins, in different proportions, were also previously detected in the last few years in the water blooms occurring in Zemborzycki dam reservoir [6–8, 10]. It is known that during the bloom ageing and decay, the intracellular toxins are released into the water [5]. Although extracellular cyanotoxins are relatively fast diluted by water mixing, local high toxin concentrations may be temporally reached. That may pose a threat for water users, including people, as well as organisms living in the contaminated water.

Although there are many methods for monitoring and the toxicity evaluating of cyanobacterial water blooms, each of them has some limitations [1]. In particular, taking into consideration the complexity of environmental samples, hardly ever only a single analytical method is able to provide enough information to conclude on the overall toxicity of the mixture of all substances present in the water affected by the massive proliferation of cyanobacteria. Chemical analyses allow for identification of a limited group of compounds only, moreover they do not provide information on interactions among the substances in the mixture [3, 13]. For that reason, it seems that the best approach for the comprehensive evaluation of environmental samples is to combine different methods of analysis which give complementary data in order to obtain information closest to the real toxic potency of the tested material [3, 13].

Toxic effects induced by the studied extract and its dilutions on the organisms used in bioassays are demonstrated in Figure 1 and Figure 2. The obtained EC_{50}/IC_{50} values and TU values are shown in Table 1.

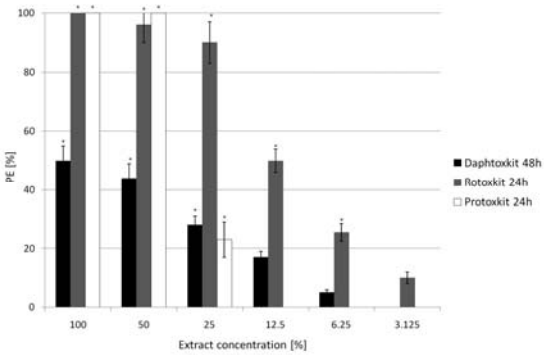

Fig. 1. Relationship between the extract concentration [%] and toxic effect PE [%], understood as death, any visible changes of behavior or population growth inhibition of the test organisms: *Daphnia magna* after 48 h of the exposure, *Brachionus calyciflorus* after 24 h of the exposure, *Tetrahymena thermophila* after 24 h of the exposure; ($\bar{x}$± SD) * – when PE > 20%, extract dilution was treated as toxic

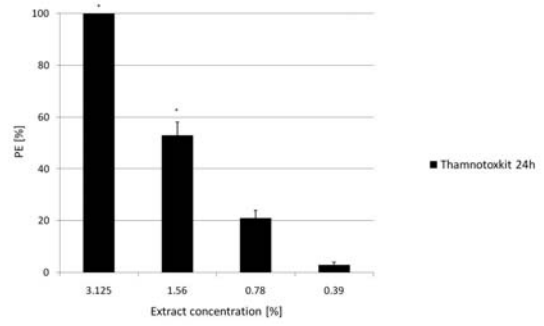

Fig. 2. Relationship between the extract concentration [%] and toxic effect PE [%], understood as death of the test organism *Thamnocephalus platyurus* after 24 h of the exposure; ($\bar{x}$±SD) * – when PE > 20%, extract dilution was treated as toxic

Tab. 1. EC_{50}/IC_{50} values and toxicity units (TU) of the studied extract obtained with the use of different invertebrates

Organism	EC_{50}/IC_{50} expressed as % of the initial extract	TU (toxicity units)
Daphnia magna 48hEC_{50}	99.5	1
Thamnocephalus platyurus 24hEC_{50}	1.2	83
Brachionus calyciflorus 24hEC_{50}	11.6	8.6
Tetrahymena thermophila 24hIC_{50}	27.7	3.6

The study showed that the extract was extremely toxic to *T. platyurus*, with 24hEC_{50} estimated to 1.2% of the initial extract concentration, which corresponded to 0.27 µg/L of MC-LR and 0.16 µg/L of Antx-a. Such high sensitivity of *T. platyurus* was also observed in the study, in which toxicity assessment of the extracts containing MC-LR, both in raw form (crude extracts) and purified on SPE column form, with a battery of 17 bioassays was conducted [4]. That susceptibility of *T. platyurus* to the impact of cyanobacterial products makes that crustacean a very sensitive and useful tool in the water bloom toxicity assessment.

Evident toxic effects of the extract were also observed on the rotifer *B. calyciflorus* (24hEC_{50} estimated to 11.6%, corresponding to 2.65 µg/L of MC-LR and 1.51 µg/L of Antx-a) and ciliate *T. thermophila* (24hIC_{50} – 27.7%, corresponding to 6.34 µg/L of MC-LR and 3.61 µg/L of Antx-a). The highest resistance to the toxic impact of the extract showed *D. magna*.

Cyanotoxins in their highly purified forms show, depending on the tested organism, several times lower toxicity, comparing to the crude extracts with the same toxin concentrations [Sierosławska, unpublished data] [14]. Discrepancies between EC_{50}/IC_{50} values of pure cyanotoxins and the crude extracts containing similar amounts of the toxins lead to the conclusion that the observed high toxicity of the extract is not only the effect of the direct activity of identified toxins. It cannot be excluded that the observed differences result from interactions between cyanotoxins present in the extracts, as well as other, not identified bioactive cyanobacterial products.

Since TU value obtained with the assay based on the *T. platyurus* reaction was within the range of $10 < TU \leq 100$, the extract was classified into the IV class, of the five class toxicity classification system [10]. This indicates that the studied sample is of the high acute toxicity.

The presence of different cyanotoxins in the extract and its high toxicity, in terms of recreational use of Zemborzycki dam reservoir, indicates the need for continuous monitoring of the water quality, and, if necessary, warning the people who use the water from the reservoir against the potential danger of poisoning.

CONCLUSIONS

- Cyanobacterial products released during the bloom-forming cell decay induce severe toxic impact on water invertebrates.

- Bioanalytical methods, as a complement to the chemical analysis, allow for the comprehensive assessment of water environment quality, answering the questions: if bioactive substances are present and what is their total toxic impact on living organisms. Moreover, as not all potentially toxic compounds may be identified with the instrumental techniques, bioassays may serve as a sensitive tool for toxin detection.
- As the reactivity of organisms used in bioassays for cyanobacterial products differs greatly, there is a need for using a set of biotests. Among other bioindicators, *T. platyurus* exhibits the highest sensitivity.

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ZASTOSOWANIE BIOTESTÓW DO OCENY TOKSYCZNOŚCI EKSTRAKTU UZYSKANEGO Z KOMÓREK SINIC

Celem pracy była ocena toksyczności ekstraktu uzyskanego z komórek cyjanobakterii obecnych w wodzie pobranej podczas zakwitu sinic z Zalewu Zemborzyckiego, z wykorzystaniem zestawu biotestów. Identyfikacja taksonomiczna wykazała w badanej próbce wysoką liczebność *Aphanizomenon flos-aquae* i *Dolichospermum* spp. (*Anabaena* spp.) oraz w mniejszym stopniu *Microcystis aeruginosa* i *Planktothrix agardhii*. Analiza HPLC ekstraktu uzyskanego z zagęszczonych komórek cyjanobakterii wykazała obecność mikrocystyny-LR w stężeniu 22,89 µg/L oraz anatoksyny-a w stężeniu 13.02 µg/L. Stopień toksyczności uzyskanego ekstraktu oceniano na podstawie reakcji organizmów testowych, z użyciem następujących biotestów: Daphtoxkit F magna (*Daphnia magna*), Thamnotoxkit F (*Thamnocephalus platyurus*), Rotoxkit F (*Brachionus calyciflorus*) oraz Protoxkit F (*Tetrahymena thermophila*) (Microbiotests Inc., Belgia). Przeprowadzone badania wykazały szczególnie silne działanie toksyczne analizowanego ekstraktu wobec *T. platyurus* (24hEC_{50} oszacowane na 1,2% ekstraktu wyjściowego). Wyraźny efekt toksyczny obserwowany był także wobec *B. calyciflorus* i w mniejszym stopniu wobec *T. thermophila*. Natomiast największą odporność na toksyczne działanie ekstraktu wykazywała *D. magna.* Na podstawie uzyskanych wartości TU (jednostek toksyczności) badany ekstrakt zaliczony został do IV klasy toksyczności, tj. o wysokiej toksyczności ostrej. Z przeprowadzonych badań wynika, że organizmy na których wykonywane są testy toksyczności wykazują zróżnicowaną wrażliwość na produkty cyjanobakterii, stąd ocena toksyczności próbek zawierających tego typu substancje powinna być prowadzona z wykorzystaniem jak najszerszego zestawu biotestów.