Evaluation of the Sensitivity of Organisms Used in Commercially Available Toxkits to Selected Cyanotoxins

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Abstract

Recently, assays based on living organisms have become a frequently used tool in toxicity assessment of environmental samples containing cyanotoxins. The aim of this study was to determine the sensitivity of different organisms from commercially available toxkits to three commonly detected cyanotoxins: microcystin-LR, cylindrospermopsin, and anatoxin-a. Cyanotoxins were used in purified forms, the highest concentration tested was 4 µg/ml. The most pronounced toxic effects were observed in the presence of cylindrospermopsin, with the lowest LC_{50} (24h LC_{50} = 0.27 µg/ml) estimated for the crustacean *Thamnocephalus platyurus*. On the contrary, anatoxin-a was found to be toxic only to a small degree, with the EC_{50} only calculated for *Daphnia magna* (24h EC_{50} = 2.09 µg/ml; 48h EC_{50} = 1.70 µg/ml). In that case the test endpoint was manifested as an inability to swim freely rather than lethality, so it was impossible to estimate LC_{50} values. In turn, the most pronounced toxic effects of microcystin-LR were observed in *D. magna* after 48-h exposure with EC_{50} estimated at 0.97 µg/ml. The lowest sensitivity to the cyanotoxins at studied concentrations was demonstrated in acute tests for the rotifer *Brachionus calyciflorus*, and to some extent the algae *Selenastrum capricornutum*. It can be concluded, therefore, that organisms used as bioindicators vary considerably in their sensitivity to cyanotoxins and the obtained EC_{50}/LC_{50} values are much higher than typical toxin concentrations in the environment.

Keywords: cyanotoxins, bioassays, microcystin-LR, cylindrospermopsin, anatoxin-a

Introduction

Contamination of water environments causes many adverse effects on water ecosystems as well as on human health and the economy. During the last few decades cyanobacteria have become one of the main problems connected with water deterioration worldwide. Cyanobacteria are photosynthetic organisms, commonly occurring around the globe, that are present in all water reservoirs. Under favorable conditions they multiply rapidly, forming water blooms that may concentrate on the water surface as scum [1]. During water blooms, except for changes in water taste and aroma, a decrease in oxygen concentration, disturbances in aquatic organism interactions, and production and release of highly toxic compounds called cyanotoxins often occur. Cyanotoxins, depending on their toxic activity, are divided into hepatotoxins (microcystins), cytotoxins (cylindrospermopsin), neurotoxins (anatoxin-a, anatoxin-a(s), saxitoxin), and dermatotoxins (lyngbyatoxin A, aplysiatoxins, debromoaplysiatoxins and endotoxin-LPS) [1].

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Cyanotoxins pose a threat not only to fish or invertebrates living in contaminated environments, but also to terrestrial animals and humans who use such water for consumption or recreational purposes. This problem is of great importance, especially in regions where water is used by humans or animals without previous treatment. For that reason, there is a need to develop a set of methods for the rapid and inexpensive detection of potential water toxicity [2].

There is a wide range of methods enabling the detection of cyanotoxins in different types of environmental samples. Among them are instrumental techniques such as high-performance liquid chromatography (HPLC) variants, gas chromatography-mass spectrometry (GC-MS), and liquid chromatography (LC-MS) [1, 3]. These methods, although very sensitive, require trained staff and time-consuming preparation of samples, and are relatively expensive. However, first of all they do not provide information on the real toxic potency of the studied materials [3, 4]. In this context, tests based on living organisms seem to be a very helpful complement to analytical methods.

There were many attempts to use different biotests in detection of cyanotoxins and the toxicity determination of cyanotoxin-containing samples [5-11]. Most of them, however, concerned hepatotoxins, including microcystins or nodularins. Only a very few studies have been performed to evaluate the impact of other cyanotoxins, e.g. anatoxin-a or cylindrospermopsin [9, 11], and information on the sensitivity of the organisms that are used in toxkits to these cyanobacterial products are scarce.

Microcystin-LR (MC-LR) and anatoxin-a (Antx-a) are among the most frequently detected cyanotoxins in Poland [12, 13], as well as in the rest of the world [14-16]. Recently, reports of the presence of cylindrospermopsin (CYN) in European water bodies, including Poland, have started to appear [17-19].

The aim of the present study was to determine the toxicity of MC-LR, CYN and Antx-a, in purified forms (commercially available standards) with the use of both acute and chronic toxicity biotests, based on five different groups of organisms belonging to various trophic levels. On the basis of the obtained results it could be possible to establish the sensitivity of the used organisms in relation to the studied toxins and to select a set of bioassays that demonstrate the greatest sensitivity to a particular type of cyanotoxin.

Experimental Procedures

Cyanotoxins

The following standards of cyanotoxins were used in this study: MC-LR ($C_{49}H_{74}N_{10}O_{12}$); CYN ($C_{15}H_{21}N_5O_7S$) purchased from Alexis Biochemicals, Switzerland; and Antx-a in the form of (±)-anatoxin-a fumarate ($C_{10}H_{15}NO \cdot C_4H_4O_4$), purchased from Tocris Bioscience, UK. The highest concentration used, 4 µg/ml, was obtained by the dilution of the toxin in an appropriate medium. Then a series of 1:1 dilutions was prepared.

Toxicity Determination

In order to assess the effects of the cyanotoxins, three acute toxicity tests: Daphtoxkit F magna [20], Thamnotoxkit F [21], Rotoxkit F Acute [22], as well as two chronic toxicity tests: Protoxkit F [23] and Algaltoxkit F [24], were used. All toxits were purchased from Microbiotests, Belgium. Producer protocols were strictly followed, including verification of culture media pH and the quality of controls. The tests were considered valid if the number of dead and immobile organisms in the controls did not exceed 10% (Daphtoxkit F, Thamnotoxkit F, Rotoxkit F), the decrease in the optical density of the controls after 24 h was of at least 40% (Protoxkit F), and the average growth rate in the controls was at least 0.92 per day (Algaltoxkit F). At least two independent replications of each test were conducted. The organisms used in the assays were obtained from the toxkits producer in cryptobiotic state as a part of the tests and were activated prior to studies.

The Daphtoxkit F magna assay was used to evaluate toxicity of the cyanotoxins to the crustacean *Daphnia magna*. After 24 h and 48 h exposure at 20ºC, in the dark, the changed behaviour, manifested by the evidently changed swimming pattern, or death of the organisms was recorded for both EC and LC estimation. Each performance of the test consisted of four replicates, with five organisms in each. The Thamnotoxkit F assay was used to evaluate cyanotoxin toxicity to the crustacean *Thamnocephalus platyurus*. The number of death organisms (not moving during 10 sec of observation) was recorded after 24 h exposure on the toxins, at 25ºC in the dark. Each test consisted of three replicates, with ten organisms in each. The Rotoxkit F acute assay was performed to evaluate cyanotoxin toxicity to the rotifer *Brachionus calyciflorus*. Five rotifers per well were used and six wells were prepared for each replicate. The organisms that did not exhibit any movement for 5 sec after gentle plate agitation were scored. For easier observation, the plate was placed on a black pad and examined with incidental illumination. Chronic toxicity of the cyanotoxins was determined by the 24 h growth inhibition Protoxkit F kit, along with the use of ciliate *Tetrahymena thermophila*. The assay is a multi-generation growth test that includes 5 or 6 ciliate generations. The inhibition of the turnover of the substrate into ciliate biomass in the presence of the tested cyanotoxins, reflecting the degree of growth inhibition, was recorded by the turbidity measurement at 440 nm (BioRad, 550). The exposure was conducted at 30ºC in the dark. Each test consisted of two replicates. A growth inhibition bioassay, Algaltoxit F, was used to assess the effects of MC-LR and Antx-a on the algae *Selenastrum capricornutum*. CYN was not tested in that assay. Algae were cultivated in 10-cm cuvettes at 23ºC, 36 µE/m²s. OD readings at 670 nm were taken after 24 h, 48 h, and 72 h using a Unicam spectrophotometer. For each of the two tested cyanotoxins, three cuvettes were prepared for each concentration.

Table 1. Estimated LC_{50}, EC_{50}, IC_{50}, and ErC_{50} values with 95% confidence intervals for five different organisms exposed to three pure cyanotoxins: microcystin-LR (MC-LR), cylindrospermopsin (CYN), and anatoxin-a (Antx-a).

Biotest	Organism	Cyanotoxin concentration [µg/ml]		
		MC-LR	CYN	Antx-a
Daphtoxkit 24h LC_{50}	*Daphnia magna*	2.78 (1.78-6.86)	> 4	> 4
Daphtoxkit 48h LC_{50}	*Daphnia magna*	1.46 (0.97-2.12)	0.89 (0.60-1.55)	> 4
Daphtoxkit 24h EC_{50}	*Daphnia magna*	1.22 (0.83-1.68)	> 4	2.09 (1.75-2.50)
Daphtoxkit 48h EC_{50}	*Daphnia magna*	0.97 (0.67-1.32)	0.89 (0.60-1.55)	1.70 (1.37-2.13)
Thamnotoxkit 24h LC_{50}	*Thamnocephalus platyurus*	1.85 (1.60-2.15)	0.27 (0.249-0.30)	> 4
Rotoxkit 24h EC_{50}	*Brachionus calyciflorus*	> 4	> 4	> 4
Protoxkit 24h IC_{50}	*Tetrahymena thermophila*	1.91 (1.53-2.20)	0.48 (0.32-0.93)	> 4
Algaltoxkit 72 hErC_{50}	*Selenastrum capricornutum*	> 4	not tested	> 4

Toxicity Data Analysis

Toxicity data obtained from biotests were expressed as the percentage of the toxic effect (PE) in comparison to the control. If the PE value was ≤ 20%, the dilution was treated as non-toxic [25]. The LC_{50} (lethality) and EC_{50} (immobilization) values and their 95% confidence limits were determined by probit analysis using the USEPA Probit Analysis Program, version 1.5. The median inhibitory concentration of ciliate growth (IC_{50}) values was obtained by a linear interpolation method. Inhibition percentages of the algal growth rates in the cyanotoxin concentrations in comparison to the growth rate of the control were calculated to determine ErC_{50} with the use of the Algaltoxkit spreadsheet.

Results

Estimations of EC_{50}, LC_{50}, IC_{50}, and ErC_{50} are presented in Table 1.

Daphtoxkit

The toxic effects of the studied cyanotoxins on *D. magna* are shown in Figs. 1.A. and 1.B. The most pronounced effects on *D. magna* were observed in the presence of MC-LR. At the highest used concentration (4 µg/ml) the whole exposed population demonstrated significant signs of toxicity, manifested both as an inability to swim freely and as high lethality (PE=100%). PE reached the values below 20%, considered as non-toxic, at 0.5 µg/ml in both, 24-h and 48-h, experiments. After organism exposure to Antx-a, lethal effects were rare but evident concentration-dependent disturbances in swimming behaviour were recorded, allow-ing the calculation of the EC_{50} value (24 h ECs was 2.09 µg/ml and 48h ECs was 1.07 µg/ml). In that regard, at the highest concentration used, the toxin was found to have a similar toxic potency to MC-LR. The final toxin studied, CYN, exerted only a slight influence on *D. magna* after 24 h exposure (at 4 µg/ml PE reached 30%); however, after 48 h the mortality of the tested organisms was very high, and EC_{50} coincided with LC_{50} in that case (0.89 µg/ml).

Thamnotoxkit

The impact of studied cyanotoxins on *T. platyurus* are shown in Fig. 1.C. Among three tested cyanotoxins, *T. platyurus* was shown to be the most sensitive to CYN. Complete mortality of the exposed population was seen up to the concentration of 0.5 µg/ml. Moreover, in the case of CYN, the Thamnotoxkit was also the most sensitive of the toxkits used. Severe concentration-dependent response also was recorded in MC-LR-exposed organisms, with PE at the highest used concentration reaching 90%. Similarly, high toxicity also was observed at 2 µg/ml, while at the next tested MC-LR concentration, 1 µg/ml, no such effects were detected. Low or no toxicity, with PE not exceeding 20%, was seen after crustacean exposure to Antx-a at the concentrations used.

Rotoxkit

The toxic effects of studied cyanotoxins on *B. calyciflorus* are shown in Fig. 1.D. Compared to the other bioassays used, the toxkit based on rotifers was found to be the least sensitive to the studied cyanotoxins, with EC_{50} values higher than the highest toxin concentration used in the study. In any case, PE did not exceed 20%.

Protoxkit

The influence of the studied cyanotoxins on *T. thermophila* are shown in Fig. 1.E. Ciliates turned out to be slightly less sensitive to MC-LR than *T. platyurus*, but the toxin at the two highest concentrations caused *T. thermophila* growth inhibition of approximately 80-75% in comparison to the control. In turn, the strongest inhibition of the population growth after the 24 h incubation period was observed as a response to the exposure to CYN. Toxic effects expressed as PE were higher that 20% up to a con-

centration of 0.5 µg/ml, and the EC_{50} in this case was estimated to 0.48 µg/ml. CYN at 0.25 µg/ml occurred to be not toxic. Low toxicity with IC_{50} >4 µg/ml was seen after protozoan exposure to Antx-a.

Algaltoxkit

The effects of MC-LR and Antx-a on *S. capricornutum* are presented in Fig. 1. F. After algae incubation in the presence of MC-LR, some concentration-dependent inhibition was observed, but it did not exceed 30%, so the
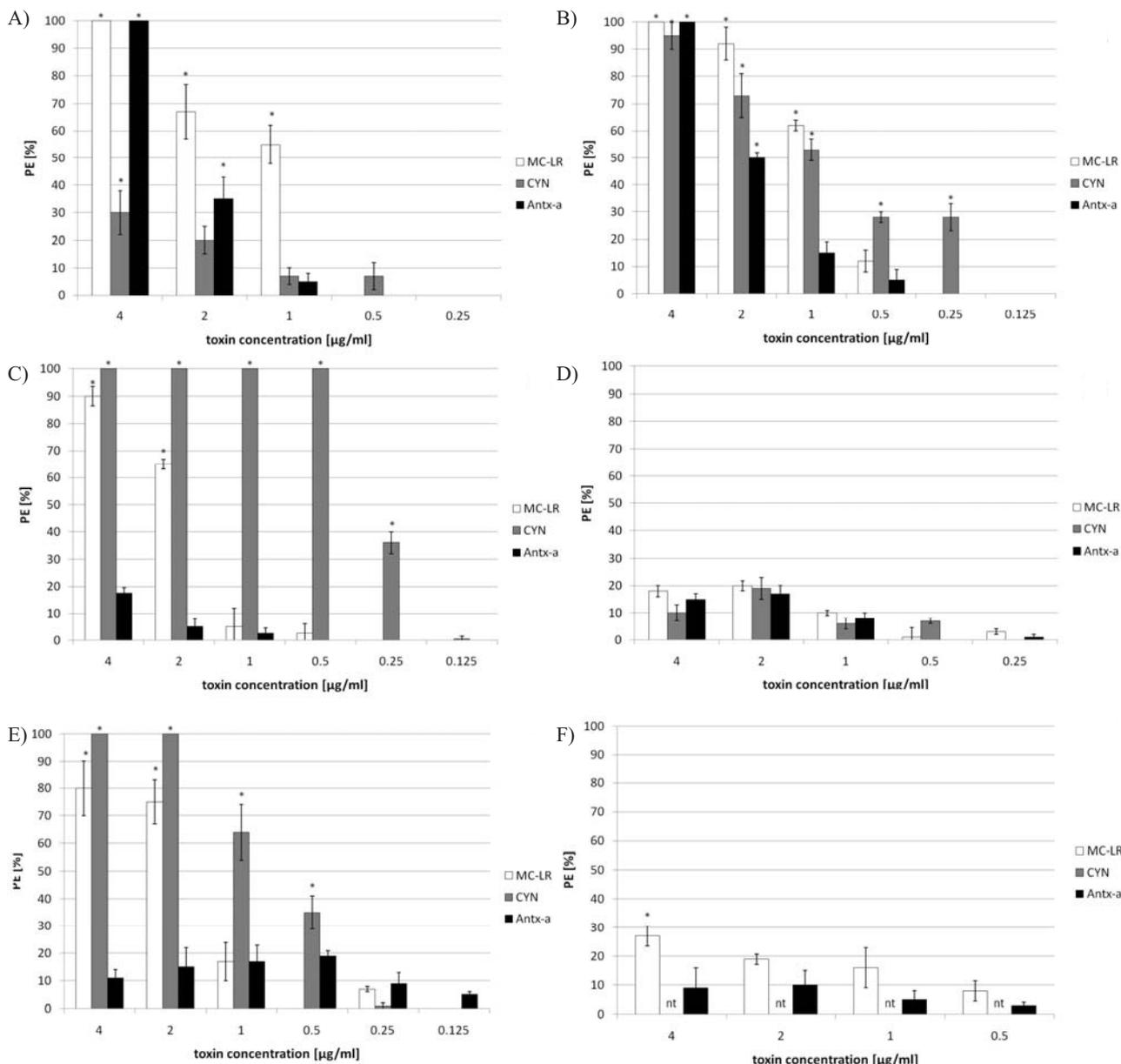

Fig. 1. Effects of three pure cyanotoxins: microcystin-LR (MC-LR), cylindrospermopsin (CYN) and anatoxin-a (Antx-a), expressed as toxic percentage effect (PE) in comparison to the control on: A) *Daphnia magna* after 24 h-exposure, calculated on the basis of any evident signs of the organism disturbances (n = 8); B) *Daphnia magna* after 48 h exposure, calculated on the basis of any evident signs of the organism disturbances (n = 8); C) *Thamnocephalus platyurus*, calculated on the basis of organism mortality (n = 6); D) *Brachionus calyciflorus*, calculated on the basis of the organism immobilization, (n = 6), E) *Tetrahymena thermophila*, calculated on the basis of population growth inhibition (n = 4); and F) *Selenastrum capricornutum*, calculated on the basis of population growth inhibition (n = 6), where n was the number of all repetitions. Results expressed as ±SD; *PE > 20% regarded as toxic; nt – not tested.

ErC_{50} was estimated to be above 4 µg/ml. Antx-a had no influence on algae growth rate at the concentrations studied, compared to the control. CYN was not tested with the use of that toxkit.

Discussion

Toxicity testing based on living organisms has become a valuable complement to chemical analyses of water contamination. Knowledge on the sensitivity of particular organisms used in bioassays to different cyanotoxins in this context seems to be of importance. It should be clearly indicated, that typical toxin concentrations in the environment seldom reach as high concentrations as EC_{50}/LC_{50} values estimated in the present study. The provisional guideline value of MC-LR in drinking water is 1 µg/l [26], and for the other two cyanotoxins guideline values in the range of 1 to 6 µg/l are suggested [14]. In turn, the recommended concentration limit for microcystins in recreational water, depending on the country, ranges from 6 µg/l to 100 µg/l [14, 27]. The amounts of cyanotoxins reaching mg/l were detected in environmental samples, but only when they were determined as a sum of both intra- and extracellular toxins in water column or a toxin content in the scum material [1, 15, 28]. The probability, that such high concentrations appear in the water may take place only when the massive cyanobacterial cell lysis occurs, but even then they have a temporary nature due to water mixing. In the case of CYN the situation is different, as the toxin is not retained in the cells but released outside, so even 70-98% of the produced toxin can be found in the water [27].

The effects of toxic cyanobacteria on organisms used in bioassays were investigated mainly with the use of crude extracts [7-9, 28] or purified crude extracts [7], which still might contain a mixture of other toxic compounds. Only a few studies were carried out with the use of highly purified toxins [5, 29]. In most cases, the bioassays provided information of overall toxicity of the tested samples, which although very informative, may not correlate with specific toxin concentrations.

In the present study it has been shown that different organisms used in bioassays possess varied sensitivity to the tested cyanotoxins, but generally toxic effects were observed at concentrations higher than those typical for natural water samples.

The results obtained indicate that Thamnotoxkit with *T. platyurus* is sensitive to CYN (LC_{50} 0.27 µg/ml) and to MC-LR (EC_{50} 1.85 µg/ml), but not to Antx-a. On the contrary, *B. calyciflorus* did not show a significantly increased abnormality rate following exposure to any of the studied toxins. These observations are consistent with the conclusions from the paper by Maršálek and Bláha [7], where strong variability in the responses of 17 different organisms to crude MC-containing extracts from cyanobacterial biomass, toxins purified from these extracts, or pigment-containing fractions were described. In that study, a similar high sensitivity of *T. platyurus* was observed after crustacean exposure to MC-LR-containing environmental samples, but also to extracts without MCs, containing pigment

fraction only. The LC_{50} values presented by the authors for crude extracts and for toxin fractions, both calculated for samples of high (485 µg/ml) and medium (211 µg/ml) MC-LR concentration, were lower by approximately an order of value than those obtained in the present study. Acute toxicity of a microcystin-containing extract to *T. platyurus* also was studied by Keil et al. [30], and LC_{50} was found to be as low as 0.46 µg/ml. That discrepancy between the toxicity level of pure MC-LR determined in the present study and much stronger impact of microcystin-containing extracts of the same toxin content, as well as the relatively high toxicity of microcystin-free extracts [7, 30], indicates the presence of other toxic, cyanobacterial-derived compounds in studied environmental samples. Similarly, Törökné et al. [10] suggested that studied crude extracts of cyanobacteria contained other components in addition to MCs, increasing their toxicity to *T. platyurus*. On the contrary, in the other study by Törökné [29], LC_{50} for pure MC-LR was reported to be as low as 0.1 µg/ml, which is much lower than that presently obtained.

In the present study, the high lethality of *T. platyurus* was induced by pure CYN, which is in accordance with the results of Törökné et al. [11], who demonstrated the high toxicity of the toxin-producing the *Cylindrospermopsis raciborskii* strain (LC_{50} = 0.14 mg/ml with 950 µg of CYN per g of biomass).

On the other hand, *T. platyurus* was shown to be very sensitive to the freeze-dried neurotoxic *Anabaena* strain and the authors' conclusion was that the organism was able to react to a neurotoxin, but no information of the Antx-a concentration in the studied sample was given [10]. However, taking into consideration that no effects after *T. platyurus* intoxication with pure Antx-a at the concentrations used were seen in the present study, the sensitivity of the crustacean to the toxin seems to be rather low.

The only organism whose reactions allowed the calculation of EC and LC values after neurotoxin exposure was *D. magna*. These findings make it necessary to revise the conclusions of our previous observations, in which a cyanobacterial crude extract exerted strong toxic effects on *D. magna*, but also on *T. thermophila* [28]. Although in the mentioned study we concluded that the inhibition was probably the result of high Antx-a concentration (1,035.59 µg/L), the low toxicity of pure Antx-a demonstrated in the present study may suggest other factors affecting the bioassay results. While in the case of the crustacean the toxic influence of the neurotoxin was confirmed, protozoan growth inhibition had to be induced by unidentified bioactive components obtained from the cyanobacterial cells.

In the present study, *T. thermophila* population growth was affected to the highest degree by CYN (IC_{50} estimated at 0.48 µg/ml). A lower sensitivity was observed towards MC-LR, with IC_{50} estimated at 5.42 µg/ml, which was slightly higher than the IC_{50} obtained for the MC-LR-containing fraction used in the study by Maršálek and Bláha [7]. Generally, data available in the literature on cyanotoxins or cyanotoxin-containing extracts on *T. thermophila* are very limited.

The most extensive effects of MC-LR were observed when *D. magna* was used, with 48 h EC_{50} estimated at 0.97 µg/ml and 48h LC_{50} at 1.46 µg/ml. The EC and/or LC values were also obtained for the other two cyanotoxins. The distinction between EC and LC was made because of evident differences in toxin concentrations at which clearly toxic effects, manifested as impairments of swimming patterns, and the lethality of the organisms were observed. Generally, cladocera-based tests were found to be the most useful in toxicity assessment, because of organism reactivity. Moreover, the tests are also the easiest to perform, which is important in routinely performed toxicity determination of high numbers of samples at the same time.

In the assay based on *S. capricornutum* growth rate, some slight inhibition at the highest MC-LR concentration was observed, but this was not distinct enough to allow for ErC_{50} estimation. The drawback of that test is the need for the continuous (72 h) use of strong illumination, which enables adequate algae growth but at the same time causes the photolytic decomposition of susceptible toxins such as Antx-a [31].

The lowest sensitivity to the cyanotoxins used was found for *B. calyciflorus*, which is in accordance with data from Maršálek and Bláha [7], concerning MC-LR-containing samples. Similarly, *B. calyciflorus* was shown to be the least sensitive organism among other rotifers exposed to pure Antx-a, which caused a reduction in reproduction rate and lifespan only at the high concentration of 5 µg/ml [32]. According to the author, the relatively low sensitivity of *B. calyciflorus* to Antx-a may reflect an evolutionary adaptation, as the rotifer is able to efficiently ingest potentially toxic cyanobacteria. That resistance of *B. calyciflorus* to toxic metabolites of cyanobacteria seems to be confirmed also by observations that lethal effects of feeding on microcystin-containing *Microcystis* cells were due to their low nutritional value but not cyanotoxin presence [33]. When choosing a set of bioassays for cyanotoxin toxicity evaluation, it seems more appropriate to use other, more sensitive representatives of rotifers, such as *Synchaeta pectinata* [32], and the survival tests should be completed or even replaced with the reproduction tests.

Conclusions

1. The EC_{50}/LC_{50} values obtained in the present study are much higher than typical toxin concentrations in the environment, which makes tested toxkits not well suited for toxin detection in water samples. However, based on the literature data on cyanobacterial crude extract toxicity assessment, it seems that they may be a good tool in overall toxicity evaluation.
2. Among tested cyanotoxins, CYN seemed to exhibit the highest biological activity toward most of the studied organisms, with the lowest LC_{50} obtained for *T. platyurus*.
3. As the organisms used in the assays vary considerably in their sensitivity to cyanotoxins, it is not possible to

indicate one of the highest susceptibility. It is suggested that a battery of bioassays is needed in toxicity assessment of cyanotoxin-containing samples.

Acknowledgements

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