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Screening of cyanobacterial cultures originating from different environments for cyanotoxicity and cyanotoxins

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Abstract

Eighty cultures from the Novi Sad Cyanobacterial Culture Collection (NSCCC) were screened for toxicity with *A. salina* bioassay and for common cyanobacterial toxins, microcystins/nodularin (MCs/NOD) and saxitoxin (STX), with ELISA assays. The results show that 22.5% (11) of the investigated cyanobacterial cultures in exponential phase exhibited toxicity in the *A. salina* bioassay and 38.7% (31) produced MCs/NOD and/or STX. However, the findings in the two methods applied were contradictory. Therefore, *A. salina* bioassay was repeated on 28 cultures in stationary growth phase, which were positive in ELISA assays but not in the initial *A. salina* bioassay. Seven more cultures exhibited cell-

bound toxicity, and only one extracellular toxicity. The observed difference in the toxicity indicates that cyanobacterial growth phase could affect the screening results.

The findings also varied depending on the environment from which the cultures originated. In the initial screening via bioassay, 11.8% (6 cultures out of 51) from terrestrial and 17.2% (5 out of 29) from aquatic environment showed cell-bound toxicity. Furthermore, based on the ELISA assay, 31.4% (16) of the cultures from terrestrial ecosystems were positive for the presence of the investigated cyanotoxins, and 51.7% (15) from aquatic ecosystems. Based on all results, more frequent toxin production was observed in cultures originating from aquatic environments. On the other hand, the group of terrestrial cultures that originated from biological loess crusts were basically non-toxic.

The discrepancies in the results by two different methods indicates that the use of several complementary methods would help to improve the assessment of cyanobacterial toxicity and cyanotoxin analyses.

Keywords: cyanobacteria, NSCCC, *Artemia salina* bioassay, ELISA, microcystin (MC), nodularin (NOD), saxitoxin (STX)

1. Introduction

Cyanobacteria are photosynthetic prokaryotes which inhabit a wide range of aquatic and terrestrial environments throughout the world. They have existed for approximately 2.8-3.5 billion years, and are still to this day one of the most important photosynthetic organism groups on the planet (Schopf and Walter, 1982; Olson, 2006; Whitton 2012). Even though over 2600 cyanobacterial species have been described so far, it is believed that many more species still remain unknown (Nabout et al., 2013). Biodiversity of the known and collected

51 species or strains can be preserved in cyanobacterial culture collections which represent
52 important repositories and “live gene banks” that can be used for studies of cyanobacterial
53 components or metabolites, as well as ecology, toxicology, and possible biotechnological and
54 medicinal use of these microorganisms.

55 Cyanobacteria can produce numerous bioactive secondary metabolites including
56 cyanobacterial toxins (cyanotoxins). Strong evidence of the deleterious effects of cyanotoxins
57 on other organisms including humans is continuously emerging (Falconer, 1998; Kuiper-
58 Goodman et al., 1999; Carmichael et al., 2001; Stewart et al., 2008; Saqrane et al., 2009; Peng
59 et al., 2010; Žegura et al., 2011; Drobac et al., 2016, 2017; Svirčev et al., 2013, 2014, 2015,
60 2017a). Based on their target organs, cyanotoxins can be divided into several groups such as
61 hepatotoxins (eg. microcystin - MC) and nodularin - NOD) and neurotoxins (eg. saxitoxin -
62 STX). MCs are probably the most widespread and the most studied cyanotoxins, with rich
63 structural variety, encompassing over 240 variants (Spoof and Catherine, 2017), and new
64 analogues are still being discovered.

65 As cyanotoxins are regarded as an emerging threat, numerous methods for their
66 detection, identification and quantification have been developed (Kaushik and
67 Balasubramanian, 2013; Meriluoto et al., 2017). However, current routine methods (such as
68 LC-MS, ELISA, bioassays) are not capable of detecting all types and variants of
69 cyanotoxins. While instrumental analysis methods are quite accurate, they are also expensive,
70 laborious and can only detect certain toxins, depending on available standards. On the other
71 hand, test kits and bioassays are sometimes cheaper and provide quick results, but the kits
72 detect groups of toxins and suffer from some degree of unwanted cross-reactivity, while
73 bioassays are not specific/sensitive enough.

74 Current research emphasis is primarily placed on cyanobacteria in aquatic ecosystems,
75 and little is known regarding cyanobacteria and their toxicity in terrestrial ecosystems. The

aim of this paper was to a) investigate the occurrence of toxicity and different cyanotoxins in cyanobacterial cultures from the Novi Sad (Serbia) Cyanobacterial Culture Collection (NSCCC); b) compare the results obtained from cultures originating from terrestrial and aquatic environments; and c) assess the reliability of *Artemia salina* bioassay and enzyme-linked immunosorbent assay (ELISA) for the detection of toxic secondary metabolites of cyanobacteria.

2. Material and methods

During this investigation, 80 cyanobacterial cultures NSCCC were assessed with an *A. salina* bioassay, and screened for selected cyanotoxins (MCs/NOD and STX) by ELISA. Cultures were isolated from various aquatic (29) and terrestrial environments (51) from Serbia and cultured at the Department of Biology and Ecology in Novi Sad. Cyanobacteria from the cyanobacterial culture collection NSCCC were cultivated in 250 mL Erlenmeyer flasks with BG-11 medium (Rippka et al., 1979) under illumination by white fluorescent light (50 μmol photons m^{-2} s^{-1}) with a 12 hours photoperiod at 22–24±1 °C. Most investigated cultures belonged to the genera *Nostoc, Anabaena*, *Phormidium, Leptolyngbya, Jaaginema, Chroococcus* and *Planktolyngbya.*

The *A. salina* bioassay was conducted on two separate occasions: firstly, as an initial toxicity screening during the culture's exponential phase (on the 28th day of cultivation), and secondly, for the toxicity screening during the stationary phase. For the first screening, 20 mL of each cyanobacterial culture were filtered through filters. Filters containing the biomass (cell-bound toxin) were then air-dried overnight at 37°C. The dried filters were extracted with 75% (v/v) methanol for 24 h, sonicated, and the extracts were centrifuged. The supernatants were collected and, after an overnight evaporation in a microtiter plate at 37°C, used for

bioassay. Toxicity of cultures was assessed using *A. salina* larvae according to Kiviranta et al., (1991), and was expressed as the difference (%) between mortalities in the tested and control samples.

Analyses with the two ELISA assays followed, where about 2 mL of each cyanobacterial cultures in the stationary phase were freeze-thawed and sonicated to ensure cellular decomposition and release of intracellular content. The extract was then centrifuged (NF 800 R, Nüve, Turkey) at 2348 × *g* for 15 min and the supernatant was used in two assays. The Microcystins-ADDA ELISA and Saxitoxin ELISA (Abraxis LLC, USA) are immunoassays for the quantitative and sensitive congener-independent detection of MCs/NOD and STX, respectively. The ELISA plates were read using a microplate reader (Asys Expert Plus UV, Biochrom, UK).

In 28 the cultures which were positive in the ELISA assays but not in the initial *A. salina* bioassay, a second bioassay for cell-bound and extracellular toxicity was performed again in the stationary growth phase. For the second bioassay, 20 mL of cyanobacterial cultures were filtered and the preparation of the cell-bound fraction was the same as in the first bioassay. However, the filtrate (extracellular part) was also collected from cultures in stationary phase and used as such for the bioassay.

3. Results

The assessment of acute cell-bound toxicity after 48 h in the 80 tested cultures from the exponential growth phase using the *A. salina* bioassay showed no or low toxicity (<50% mortality) in 69 cultures. Medium or high toxicity (≥50% mortality) was detected in 11 cultures, six originating from terrestrial (genera *Anabaena*, *Nostoc, Phormidium,*

125 *Synechocystis*) and five from aquatic (genera *Nostoc, Phormidium, Microcystis*, and one
126 mixed culture) environments (Table 1).
127
128 **Table 1** Results from *Artemia salina* bioassay and ELISA assay

No.	Code	Genus	*Artemia salina* bioassay	ELISA assay	
			CB exponential phase (48 h)	**MC/NOD**	**STX**
1.	T1	*Anabaena* sp.	-	-	-
2.	T2	*Anabaena* sp.	-	-	-
3.	T3	*Anabaena* sp.	-	-	-
4.	T4	*Anabaena* sp.	-	-	-
5.	T5	*Anabaena* sp.	-	-	-
6.	T6	*Anabaena* sp.	-	-	-
7.	T7	*Anabaena* sp.	-	-	-
8.	T8	*Anabaena* sp.	+	-	-
9.	T9	*Anabaena* sp.	+	-	-
10.	T10	*Anabaena* sp.	-	+	+
11.	T11	*Anabaena* sp.	-	+	-
12.	T12	*Anabaena* sp.	-	+	-
13.	T13	*Anabaena* sp.	-	+	-
14.	T14	*Anabaena* sp.	-	+	-
15.	T15	*Calothrix* sp.	-	-	-
16.	T16	*Chroococcus* sp.	-	-	-

17.	T17	*Chroococcus* sp.	-	-	-
18.	T18	*Chroococcus* sp.	-	-	-
19.	T19	*Chroococcus* sp.	-	-	-
20.	T20	*Chroococcus* sp.	-	-	-
21.	T21	*Chroococcus* sp.	-	-	+
22.	T22	*Leptolyngbya* sp.	-	-	-
23.	T23	*Leptolyngbya* sp.	-	-	-
24.	T24	*Leptolyngbya* sp.	-	-	-
25.	T25	*Leptolyngbya* sp.	-	-	-
26.	T26	*Leptolyngbya* sp.	-	+	-
27.	T27	*Nostoc* sp.	-	-	-
28.	T28	*Nostoc* sp.	-	-	-
29.	T29	*Nostoc* sp.	-	-	-
30.	T30	*Nostoc* sp.	-	-	-
31.	T31	*Nostoc* sp.	-	-	-
32.	T32	*Nostoc* sp.	-	-	-
33.	T33	*Nostoc* sp.	-	-	-
34.	T34	*Nostoc* sp.	-	-	-
35.	T35	*Nostoc* sp.	-	-	-
36.	T36	*Nostoc* sp.	-	-	-
37.	T37	*Nostoc* sp.	-	-	-
38.	T38	*Nostoc* sp.	-	-	-
39.	T39	*Nostoc* sp.	-	-	-
40.	T40	*Nostoc* sp.	-	-	-
41.	T41	*Nostoc* sp.	+	-	-

42.	T42	*Nostoc* sp.	-	+	+
43.	T43	*Nostoc* sp.	+	-	+
44.	T44	*Nostoc* sp.	-	+	-
45.	T45	*Nostoc* sp.	-	+	-
46.	T46	*Nostoc* sp.	-	+	-
47.	T47	*Nostoc* sp.	-	+	-
48.	T48	*Nostoc* sp.	-	+	-
49.	T49	*Nostoc* sp.	-	+	-
50.	T50	*Phormidium* sp.	+	+	+
51.	T51	*Synechocystis* sp.	+	-	-
52.	A1	*Anabaena* sp.	-	-	-
53.	A2	*Aphanizomenon* sp.	-	-	-
54.	A3	*Geitlerinema* sp.	-	+	-
55.	A4	*Gloeocapsa* sp.	-	-	-
56.	A5	*Gloeocapsa* sp.	-	-	-
57.	A6	*Gloeocapsa* sp.	-	+	-
58.	A7	*Jaaginema* sp.	-	-	-
59.	A8	*Jaaginema* sp.	-	-	-
60.	A9	*Leptolyngbya* sp.	-	+	-
61.	A10	*Leptolyngbya* sp.	-	+	-
62.	A11	*Nostoc* sp.	-	-	-
63.	A12	*Nostoc* sp.	+	-	-
64.	A13	*Nostoc* sp.	-	+	+
65.	A14	*Nostoc* sp.	-	-	+
66.	A15	*Nostoc* sp.	-	+	-

67.	A16	*Nostoc* sp.	-	+	-
68.	A17	*Oscillatoria* sp.	-	+	-
69.	A18	*Phormidium* sp.	-	-	-
70.	A19	*Phormidium* sp.	+	-	-
71.	A20	*Phormidium* sp.	+	-	-
72.	A21	*Phormidium* sp.	-	+	-
73.	A22	*Phormidium* sp.	-	+	-
74.	A23	*Phormidium* sp.	-	+	-
75.	A24	*Planktolyngbya* sp.	-	-	-
76.	A25	*Planktolyngbya* sp.	-	-	-
77.	A26	*Planktolyngbya* sp.	-	-	-
78.	A27	*Jaaginema* sp., *Aphanotece* sp., *Pseudanabaena* sp.	-	+	-
79.	A28	*Nostoc* sp., *Leptolyngbya* sp., *Spirulina* sp.	+	+	-
80.	A29*	*Microcystis aeruginosa* PCC 7806	+	+	-

CB exponential phase - cell-bound toxicity in exponential phase

A - cyanobacterial culture originating from aquatic ecosystem

T - cyanobacterial culture originating from terrestrial ecosystem

+ cyanotoxin detected/toxicity (≥50% mortality)

- cyanotoxin not detected/no toxicity (<50% mortality)

*control (known producer of MC)

Results from the ELISA assays showed that 31 out of 80 investigated cultures produced targeted cyanobacterial toxins (Table 1). From the total number of cultures positive for cyanotoxins, 24 cultures were positive for MCs/NOD, where 11 cultures were of terrestrial and 13 of aquatic origin. Three cultures were positive for STX, two of which were of terrestrial origin (genera *Chroococcus* and *Nostoc*) and one was aquatic (*Nostoc*). In addition, four cultures were positive for both cyanotoxin groups, three of which originated from terrestrial (genera *Anabaena*, *Nostoc, Phormidium*) and one (*Nostoc*) from an aquatic environment (Fig. 1).

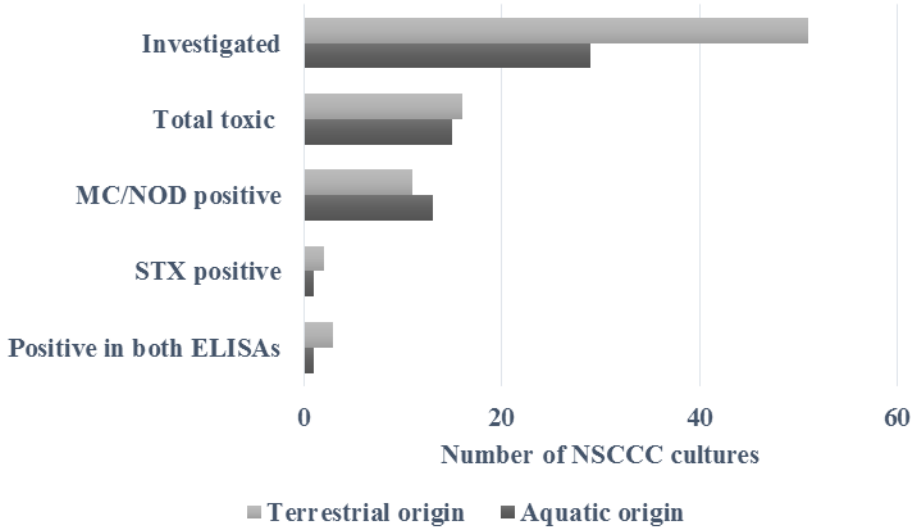
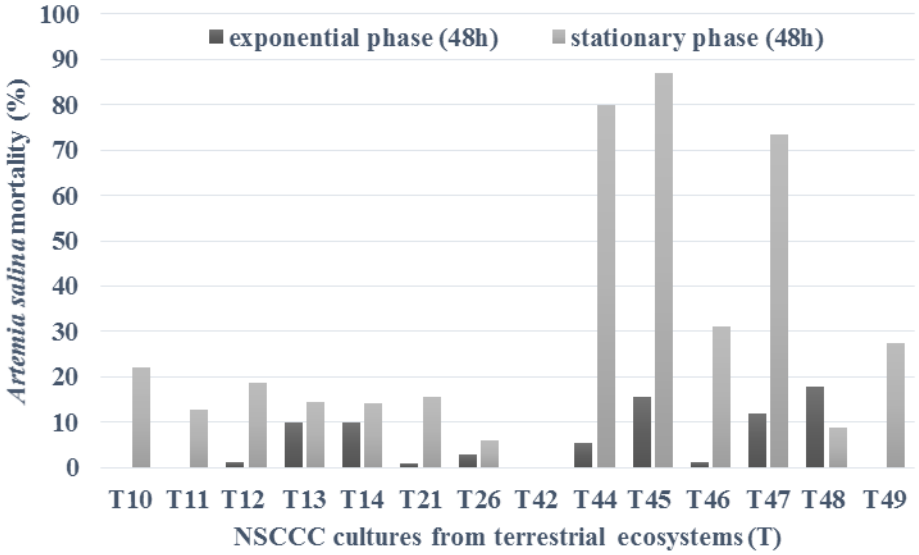


Fig. 1. Relation between cyanobacterial cultures of terrestrial and aquatic origin tested for cyanotoxins with ELISA.

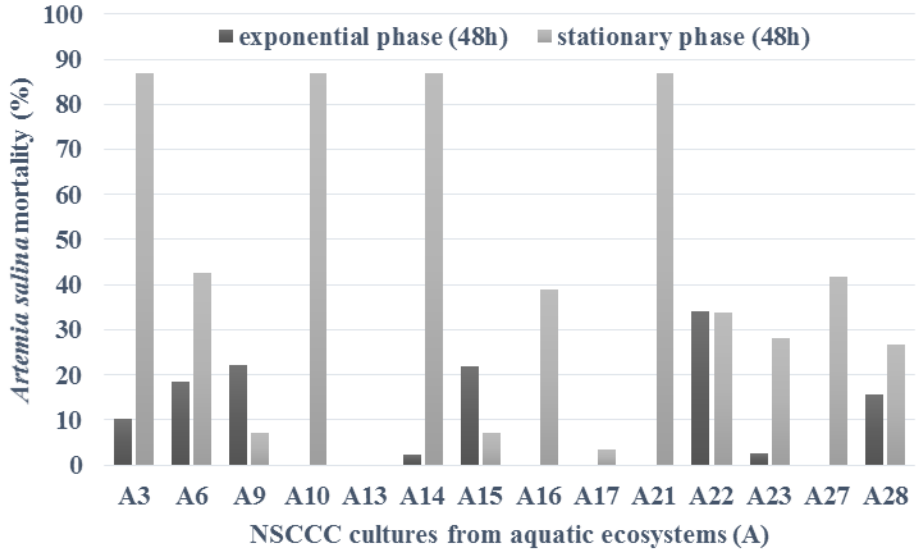
Findings from the ELISA assay and the *A. salina* bioassay on cultures from exponential phase were inconsistent. A second *A. salina* bioassay was performed on 28 cultures in the stationary growth phase. These cultures were positive in ELISA assays, but not in the initial *A. salina* bioassay. Cell-bound toxicity (>50% mortality) was observed in seven additional cultures, where three *Nostoc* cultures were from terrestrial environments (Fig. 2)

153 and four from aquatic environments (genera *Geitlerinema, Leptolyngbya, Nostoc,*
154 *Phormidium*) (Fig. 3).



155

156 **Fig. 2.** Comparison of *A. salina* bioassay results on intracellular toxicity of cyanobacterial
157 cultures from terrestrial ecosystems in different growth phases.



158

159 **Fig. 3.** Comparison of *A. salina* bioassay results on intracellular toxicity of cyanobacterial
160 cultures from aquatic ecosystems in different growth phases.

161

High extracellular toxicity (95.5% mortality) was recorded in only one culture in stationary phase (T44, *Nostoc*), and the rest of the cultures showed low or no extracellular toxicity.

4. Discussion

Secondary metabolites of cyanobacteria, both cell-bound and extracellular, can exert toxic effects in brine shrimp *A. salina* as demonstrated in a number of studies (e.g. Lindsay et al., 2006). This bioassay was previously found to be suitable for MCs (Kiviranta et al., 1991; Campbell et al., 1994), but could be also used for evaluation of STX (Park et al., 1986), anatoxin-a (Lahti et al., 1995) and cylindrospermopsin (Metcalf et al., 2002). The test organism *A. salina* can react to several cyanotoxins but also reveal (synergistic) effects of other compounds present (Lindsay et al., 2006; Pires et al., 2011). During our first initial screening via bioassay, only 11 cultures exibited intracellular toxicity, six out of 51 from terrestrial (11.8%) and five out of 29 from aquatic environment (17.2%).

The ELISA assays demostrated that some cultures produced only MCs/NOD or STX, while several cultures were positive for both groups of cyanotoxins (genera *Nostoc*, *Anabaena* and *Phormidium*). Cyanotoxins were found in different genera originating from aquatic (*Geitlerinema*, *Gloeocapsa*, *Leptolyngbya*, *Nostoc*, *Oscillatoria*, *Phormidium, Microcystis*) or terrestrial (*Anabaena*, *Chroococcus*, *Leptolyngbya*, *Nostoc*, *Phormidium*) ecosystems. Many cyanobacterial species and strains are toxigenic, i.e. able to produce cyanotoxin(s). Most research has been related to aquatic environments. Several authors have confirmed that the genus *Nostoc* is known for the production of both MCs and STXs (Sivonen et al., 1990; Sivonen et al., 1992a; Oksanen et al., 2004; Silva et al., 2014). Similar findings were reported for the genus *Anabaena* (Sivonen et al., 1992b; Namikoshi et al., 1992a, 1992b; Rapala et al.,

187 1997; Neilan et al., 1999; Belykh et al., 2011), and *Phormidium* (Izaguirre et al., 2007; Wood
188 et al., 2010). Furthermore, the production of MCs has been observed in the genera *Microcystis*
189 (Eloff and Van der Westhuizen 1981; Kiviranta et al., 1992; Luukkainen et al., 1994; Lyra et
190 al., 2001), *Leptolygbya* (Silva et al., 2014), *Oscillatoria* or *curr. Planktothrix* (Lindholm and
191 Meriluoto, 1991; Luukkainen et al., 1993; Fastner et al., 1999; Tonk et al., 2005),
192 *Pseudanabaena* (Oudra et al., 2001; Maršálek et al., 2003), *Chroococcus* (Neilan et al., 2008),
193 *Geitlerinema* (Gantar et al., 2009), and *Gloeocapsa* (Carmichael and Li, 2006). The present
194 investigation showed that 31.4% of the cultures originating from terrestrial ecosystems were
195 positive for the presence of cyanotoxins, and 51.7% of the cultures from aquatic ecosystems.
196 The obtained results were re-investigated with 28 cultures in the stationary growth
197 phase. Seven more cultures with cell-bound toxicity were detected and only one culture
198 exibited extracellular toxicity. These results indicate that the growth phase could have an
199 effect on the cyanotoxin production and excretion. Similar research showed that *Microcystis*
200 *aeruginosa* culture produced more MC in stationary than in exponential phase (Lyck, 2004).
201 Differences between production of toxic metabolites of cyanobacterial cultures
202 originating from terrestrial and aquatic ecosystems were noticeable. Although the same
203 genera known for cyanotoxin production occurred in both ecosystems, the detected
204 production of toxic metabolites was more common in cultures from aquatic ecosystems. There
205 is much data on the intensification and global expansion of harmful cyanobacterial blooms,
206 and effects on aquatic ecosystem health as well as transfer in food webs (Wiegand and
207 Pflugmacher, 2005; Ibelings and Chorus, 2007; Paerl and Huisman, 2009; Jančula and
208 Maršálek, 2011; O'Neil et al., 2012). Corresponding information concerning cyanotoxin
209 production in terrestrial environments is very limited, and include only one dataset for desert
210 crusts in Qatar where low concentrations of MCs and anatoxin-a(S) were detected (Metcalf et
211 al., 2012). Furthermore, there is a possibility that terrestrial cyanobacteria, if toxic, could

cause some unexplained diseases in grazing animals (McGorum et al., 2015), and, possibly, through food webs in humans. More research should be focused on terrestrial cyanobacteria and their toxicity. Among the investigated samples, eight NSCCC cultures were originating from biological loess crusts (terrestrial), and no toxicity was detected in the bioassay, while only one showed a very low concentration of STX in the ELISA assay. There is a possibility that these results are only false positives, which can occur in this assay. Similar findings were documented by Dulić et al. (2017) in research on Iranian loess crust samples, when very low toxin values and low toxicity were reported and explained as false positives. Therefore, the question arrises: why are cultures from biological loess crusts practically non-toxic and how does cyanotoxin production depend on the environment cyanobacteria inhabit?

In total, most investigated cyanobacterial cultures (42 out of 80) were non-toxic, and only 10 displayed both toxin presence and toxicity. However, a discrepancy in results was found while testing several cyanobacterial cultures: high toxicity in bioassay was found in seven cultures which were negative in the ELISA assays. It is possible that the presence of some other toxins, or other harmful secondary metabolites produced by cyanobacteria (Sivonen et al., 2010; Chlipala et al., 2011; Nagarajan et al., 2013) resulted in a high toxicity, however, such compounds were not detected in the specific ELISA assays. ELISA can provide initial screening concerning the presence of toxin groups, but should be complemented by physicochemical methods such as high-performance liquid chromatography (HPLC) for the identification of the individual chemical compounds (Carmichael and An, 1999). ELISA results should be interpreted with caution because of the possibility of falsepositives or false negatives, relatively high variability, and differential detection of some variants (Guo et al., 2017). There is also a possibility for matrix interference in ELISA (Gaget et al., 2017). Furthermore, some ELISAs can underestimate the concentration of certain MC variants (An and Carmichael, 1994). In the case of STX ELISA, the variation in cross-

reactivity and congener toxicity is problematic when interpreting the results (Gaget et al., 2017).

On the other hand, 21 cultures produced cyanotoxins in this study but did not exhibit high toxicity in the bioassay. Even though brine shrimp assay has been suggested as a valid and rapid screening method to evaluate cytotoxic activity (Solis et al., 1993; Lu et al., 2012), it has certain limitations. *A. salina* is a marine organism while the tested samples are mostly of freshwater origin, which can present a complication. Therefore, in the *A. salina* test, attention must be paid to the effects of salinity. Also, toxicity of all variants and types of cyanotoxins, as well as protease inhibitors has not been shown towards *A. salina*. Thus, the effect of other cyanotoxins on *A. salina* should be examined before this bioassay can be accepted universally (Agrawal et al., 2012). The study of Hisem et al. (2011) showed that it is likely that the toxic effect of cyanobacterial secondary metabolites mostly targets basal metabolic pathways present in mammal cells which is not manifested in *A. salina*, which could explain different findings with two methods. Furthermore, this toxicity test is not specific enough, but rather gives a value of the total acute toxicity. Accordingly, it is a supplementary method to the chemical analysis (Lu et al., 2012). Also, two methods were performed on cultures in different growth phases which can have an effect on the results.

The indicated discrepancies in results between the two methods used suggest that it is insufficient to assess toxicity of cyanobacteria using solely the brine shrimp bioassay or monitor presence of cyanotoxins merely with ELISA assay. Future investigations should be performed together with a more sensitive and precise methods, especially when estimating the possible health risk for humans. However, methods such as high-performance liquid chromatography (HPLC) or liquid chromatography-mass spectrometry (LC-MS) also have some major drawbacks. They require special and often very demanding sample preparation, adequate equipment and skilled personnel, which slows down and complicates the process of

cyanotoxin detection. In addition, they are expensive and limited in the number of cyanotoxins which can be tested due to the lack of suitable standards (Welker et al., 2002; Kubwabo et al., 2004; McElhiney and Lawton, 2005). These arguments were supported in two cases from Serbia investigating cyanobacteria and cyanotoxins. Water from the Aleksandrovac reservoir was tested with LC-MS/MS for the presence of some most common cyanotoxins (MCs, STX, cylindrospermopsin) after a *Cylindrospermopsis raciborskii* bloom and massive fish mortality, however, these well known cyanotoxins were not detected. Intrestingly, *A. salina* bioassay did show toxicity, suggesting the presence of uncharacterized toxic agents which could not be detected by LC-MS/MS without standards (Svirčev et al., 2016). Additionally, in the Užice case where reservoir for drinking water supply bloomed, toxin analyses of water showed a MC-LR concentration within the guideline values (Institute of Public Health Serbia 2014). However, *A. salina* bioassay uncovered the presence of toxic metabolites which was corroborated with LC-MS/MS analyses of water, biomass, and fish where other MC variants were detected (Svirčev et al., 2017b).

There is no "gold" standard when it comes to cyanotoxin detection. Perhaps the best approach would be to choose an appropriate method/methods based on the needs (cost, rapidity and reliability) and then use it consistently so that results could be comparable (Gaget et al., 2017). Furthermore, the shortcomings of singular methods should be ovecame in order to improve the assessment of cyanobacterial and cyanotoxin effects on organisms and ecosystem in general.

5. Conclusions

The investigation on 80 cyanobacterial cultures from the NSCCC showed that 17 cultures exhibited toxicity in *A. salina* bioassay and 31 cultures produced MCs/NOD and/or

STX in ELISA assays. It was observed that the growth phase could have an effect on the results. Furthermore, the toxicity/toxin presence also varied depending on the environment from which the cultures originated. More frequent toxin production was observed in cultures originating from aquatic environments compared to the cultures with terrestrial origin. One of the important findings in this research was the toxicity and production of toxins in cyanobacteria of terrestrial origin, while the group of terrestrial cultures that originated from biological loess crusts were basically non-toxic. The inconsistency in the results obtained within and between two selected methods indicated that it is advisable to use several complementary methods in order to gain more reliable results.

Conflict of interest

The authors have declared no conflict of interest.

Ethical statement

The authors agree with the Ethical Guidelines for Journal Publication. The study was carried out and the manuscript written taking into consideration these guidelilnes. All authors contributed to this manuscript and have approved the final article.

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