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RNDr. Thesis

# Cyanobacterial cytotoxicity versus toxicity to brine shrimp *Artemia salina*

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## Annotation

Heterocytous cyanobacteria from various habitats were screened for toxicity to brine shrimp *Artemia salina* and the murine lymphoblastic cell line Sp/2 in order to compare these two testing models for evaluation of risk posed by cyanobacteria to human health. Methanol extracts of biomass and cultivation media were tested for toxicity and selected extracts were fractionated to determine the active fraction. Comparison of these two toxicological models have shown that the toxic effect of cyanobacterial secondary metabolites mostly targets basal metabolic pathways present in mammal cells and so is not manifested in *A. salina*. We conclude that it is insufficient to monitor cytotoxicity of cyanobacteria using only the brine shrimp bioassay as was usual in the past, since cytotoxicity is a more frequent feature in cyanobacteria in comparison with toxicity to *A. salina*. We suggest that in vitro mammal cells be used for these purposes.

## Anotace

V rámci předkládané práce byl zkoumán toxický efekt heterocytózních sinic k mořskému bezobratlému *Artemia salina* a myším lymfocytům Sp/2 za účelem porovnání těchto dvou testovacích modelů a jejich využití ke zjišťování rizik, které představují sinice pro lidské zdraví. Biomasa i kultivační médium byly extrahovány v methanolu, testovány na toxicitu a následně frakcionovány pro zjištění aktivní frakce. Z porovnání těchto dvou modelů se zdá pravděpodobné, že toxický efekt sekundárních metabolitů sinic působí zejména na některé základní metabolické děje v savčích buňkách a proto se neobjevuje v *A. salina*. Bylo prokázáno, že je nedostatečné používat jako testy cytotoxicity pouze test na *A. salina* jako tomu bylov minulosti, protože cytotoxický efekt se u sekundárních metabolitů sinic vyskytuje mnohem častěji než toxický efekt na *A. salina*. Navrhujeme, aby se pro tento účel používaly spíše samotné savčí buňky.

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České Budějovice, 15th December Daniel Hisem

## Declaration of originality

Coauthors listed bellow confirm that Daniel Hisem contributed substantially to measurements and writing the article: Cyanobacterial cytotoxicity versus toxicity to brine shrimp *Artemia salina.* Coauthors also acknowledge Daniel Hisem as first author oh the publication.

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# Content

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# Cyanobacterial cytotoxicity versus toxicity to brine shrimp *Artemia salina*

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ABSTRACT

Heterocytous cyanobacteria from various habitats were screened for toxicity to brine shrimp *Artemia salina* and the murine lymphoblastic cell line Sp/2 in order to compare these two testing models for evaluation of risk posed by cyanobacteria to human health. Methanol extracts of biomass and cultivation media were tested for toxicity and selected extracts were fractionated to determine the active fraction. We found a significant toxic effect to *A. salina* and to Sp/2 cells in 5.2% and 31% of studied extracts, respectively. Only 8.6% of the tested strains were highly toxic to both *A. salina* and the Sp/2 cell line, and only two of the tested strains were toxic to *A. salina* and not to the murine cell line. Therefore, it is likely that the toxic effect of cyanobacterial secondary metabolites mostly targets basal metabolic pathways present in mammal cells and so is not manifested in *A. salina*. We conclude that it is insufficient to monitor cytotoxicity of cyanobacteria using only the brine shrimp bioassay as was usual in the past, since cytotoxicity is a more frequent feature in cyanobacteria in comparison with toxicity to *A. salina*. *A. salina* toxicity test should not be used when estimating the possible health risk for humans. We suggest that in vitro mammal cells be used for these purposes.



## 1. Introduction

The toxicity of cyanobacterial secondary metabolites and its ecological implications have been widely studied over the last few years (e.g. Blom et al., 2006; Ferrão-Filho et al., 2000; Gademann and Portman, 2008; Lürling and Beekmann, 2006; Sarnelle and Wilson, 2005; Wilson et al., 2006). Some of the cyanobacterial metabolites (microcystins, anatoxin-a(s)) were found to cause severe health problems in humans and animals and thus their occurrence in drinking water is currently monitored in many countries (e.g. Chorus and Bartram, 1999). However, since a wide range of structural variants of cyanobacterial metabolites have been described (Van Wagoner and Drummond, 2007; Welker et al., 2006), it is highly probable that some toxic structures evade attention. Moreover, it is estimated that the chemical structure of only about 30% of cyanobacterial peptides is known (Hrouzek et al., 2010; Welker and vön Dohren, 2006), and in an even lower proportion the biological effect is known. Thus it is extremely important to employ a complex testing system to evaluate possible health risks for humans.

For the screening of cyanobacterial toxicity, model organisms such as *Artemia salina* or *Daphnia* spp. are usually used. An *A. salina* assay has also been suggested as a valid

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method to evaluate the cytotoxic activity of plant extracts (Solis et al., 1993) and as a rapid preliminary screening method for toxic cyanobacteria (Lahti et al., 1995). Some published data have suggested a good correlation between the toxic activity in the brine shrimp assay and the cytotoxicity against some tumor cell lines (Anderson et al., 1991) and hepatotoxic activity (Kiviranta et al., 1991). The brine shrimp assay is, therefore, usually used as a low-cost and easily achievable cytotoxicity test replacing cell lines assays (Piccardi et al., 2000). However, there have been several studies in recent years that present contrary results (Berry et al., 2004; Jaki et al., 1999; Mian et al., 2003).

The inhibitory or even lethal effects of cyanobacterial metabolites on invertebrates have been shown in numerous studies. For instance, the cyanobacterial peptides microviridin J and microcystin were found to be toxic to eukaryotic organisms such as *Daphnia pulicaria* and *Daphnia galeata* (Rohrlack et al., 1999, 2004). Further, strong lethal effects on invertebrates for a number of non-peptide compounds such as cryptophycin, tolytoxin, calothrixins and pahayakolide have been found (Berry et al., 2004; Biondi et al., 2004; Rohrlack et al., 1999, 2004, 2005; Agrawal et al., 2005). However, no data on cytotoxicity to mammal cell lines and to *A. salina* are available for many cyanobacterial compounds.

The main aim of this study was to compare the toxicity of 63 crude extracts (biomass and culturing media) from cyanobacteria from various environments to the Sp/2 cell line and the model invertebrate *A. salina*, in order to find out whether results of these tests can be correlated and which of these tests should be used for the evaluation of potential health risks to humans. We also determined if the toxic effect of cyanobacterial compounds is specific to complex multi-cellular organism or more general, and we compared the frequency of toxic strains among cyanobacterial strains originating from different habitats.

## 2. Materials and methods

### 2.1. Cyanobacterial strains, cultivation and extract preparation

A total of 63 different cyanobacteria were involved in this study: 57 cultured strains of various morphospecies and 6 field samples of *Nostoc commune*. The strains originated from various habitats, including soil (18 strains), plankton (30 strains), symbiotic associations (7 strains), periphytic (4 strains) and epiphytic strains (4 strains). Soil, symbiotic, peryphitic and epiphytic strains were cultivated in Allen and Arnold medium (Arnon et al., 1974) in 300 mL cylindrical flasks, bubbled with $CO_2$ enriched air (2%) and illuminated with artificial light of PFD (photon flux density) of 280 $\mu mol\ m^{-2}\ s^{-1}$ for 2–4 weeks and harvested by centrifugation (4500 rpm, 15 min). Plankton species of the genus *Anabaena* were cultivated in WC medium (Guillard and Lorenzen, 1972) in 250 mL Erlenmeyer's flasks, illuminated with artificial light of intensity of PFD of 50 $\mu mol\ m^{-2}\ s^{-1}$ for 3–4 weeks. Biomass was harvested by centrifugation in 50 mL glass cuvettes (4500 rpm, 15 min), stored at −80 °C and lyophilized. Cultivation medium was separated from biomass after centrifugation, 50 mL of it was filtered through bacteriological filters (porosity 0.2 μm) with a water-pump to obtain cell-free medium. 200 mg of lyophilized biomass was transferred into 10 mL glass test tubes and extracted for 2 h using 70% methanol. The test tubes were centrifuged (4500 rpm, 15 min) and the supernatant then transferred into an evaporating vessel and dried in a rotation vacuum evaporator. The solid extract was re-suspended in 1 mL of 70% MeOH to get an extract of the desired concentration (200 mg of dry weight per millilitre). To obtain an extract from the medium, 50 mL of filtered media was concentrated into 2 mL 100% methanol using solid phase extraction (MCX Cartridge OASIS, Waters) and a vacuum-pump.

### 2.2. A. salina *bioassay and cytotoxicity assay*

The brine shrimp assay was done according to Lincoln et al. (1996). Cysts were incubated in artificial seawater illuminated by artificial light and gently aerated for 24 h (Metcalf et al., 2002). For toxicity tests, hatched nauplii were diluted to a concentration of 15–20 individuals $mL^{-1}$. 100 μL of extracts was transferred into a 12-well microtitrate plate and were kept in a laminar box for methanol evaporation. 50 μL of distilled water was added and sonicated to improve the dissolving of the dry extract. 0.95 mL of nauplii (15–20 individuals) was added and the numbers of living and dead individuals and unhatched cysts were counted using a stereomicroscope. Individuals were re-counted after 24 and 48 h and the percentage mortality calculated. Strains causing mortality higher than 50% were considered as highly toxic.

Murine lymphoblastic cell line Sp/2 was used for cytotoxicity testing. The cells were cultivated at 37 °C in plastic tissue culture flasks with RPMI 1640 medium with an addition of 5% fetal calf serum, 1% glutamine, and 1% antibiotic–antimycotic solution. Prior to experiments, the cells were stained with Trypan blue and counted using a haemocytometer in order to estimate viability. Only cell cultures with viability higher than 90% were used for the experiments. The cell suspension was centrifuged (1000 rpm, 10 min, 4 °C), and an adequate amount of fresh RPMI medium was added in order to obtain a concentration of $1.5 \times 10^5$ cells per well (200 μL RPMI).

Cyanobacterial extract of 10 μL was added to the wells in triplicates, and triplicates treated only by 70% methanol were left as controls. The plate was kept in an incubator (37 °C) until all the 70% methanol was evaporated. The cell suspension was then added and incubated (37 °C and 3.5% $CO_2$) for 12 h. Cell viability after exposure was estimated by MTT assay (Mosmann, 1983); 10 μL of MTT solution (4 mg $mL^{-1}$) was added and the plates were incubated for 4 h. The plates were centrifuged after the incubation (3000 rpm, 10 min) and the supernatant was removed. 200 μL of DMSO was added to dissolve formazan crystals. Test and background absorbances were measured at 590 and 640 nm, respectively. The survival of cell lines was evaluated as the ratio of treated wells' absorbance to that of the control wells, and expressed as a percentage.

### 2.3. Extract analysis

The extract composition was analysed using an HP 1100 Agilent mass spectrometer with an HP 100 MSD SL-Ion

**Table 1**
List of studied cyanobacteria strains. Place of isolation, habitat, *A. salina* mortality (B – biomass, M – medium, + represents mortality ≥ 50%) and Sp/2 cell line inhibition is shown. Different habitats are marked by abbreviations: E (epiphytic), P (periphytic), S (soil), Sy (symbiotic) and Pl (planktonic).

| Scientific name | Strain | Place of isolation | Habitat | *A. salina* mortality | | Sp/2 inh. [%] |
|---|---|---|---|---|---|---|
| | | | | B | M | Mean |
| *Nostoc* sp. | BR III[b] | Paranapiacaba-Sao Paulo/Brazil | E | + | – | 12[a] |
| *Nostoc* sp. | RQII[b] | Paranapiacaba-Sao Paulo/Brazil | E | – | – | 7[a] |
| *Nostoc* sp. | BRIIB[b] | Paranapiacaba-Sao Paulo/Brazil | E | – | n.a. | 0[a] |
| *Nostoc* sp. | BROMEL[b] | Paranapiacaba-Sao Paulo/Brazil | E | – | – | 0[a] |
| *Nostoc* sp. | LC17S01 | Alberta/Canada | P | – | – | 27 |
| *Nostoc* sp. | OSNI 32S01 | Sítinový pond/Czech Republic | P | – | n.a. | 0[a] |
| *Cylindrospermum* sp. | Hrouzek 1/2004 | Zliv/Czech Republic | P | + | n.a. | 100 |
| *Calothrix* sp. | Hrouzek 2/2005 | San Monoron-BUSRA/Cambodia | P | – | – | 30 |
| *Nostoc calcicola* | Lukešová 2/89[e] | Havana/Cuba | S | – | – | 6[a] |
| *Nostoc* sp. | Lukešová 5/96[e] | Nezamyslice/Czech Republic | S | + | – | 52[a] |
| *Nostoc muscorum* | Lukešová 2/91[e] | Nezamyslice/Czech Republic | S | + | – | 48[a] |
| *Cylindrospermum* sp. | C 24[e] | Ellesmere island/Canada | S | – | – | 60 |
| *Trichormus variabilis* | ISB 13 | Dlouhá Ves/Czech Republic | S | – | – | 62.3 |
| *Nostoc* sp. | Lukešová 7/99[e] | Germany | S | – | – | 66[a] |
| *Nostoc* sp. | Lukešová 24/97[e] | Germany | S | – | – | 0[a] |
| *Nostoc* sp. | Lukešová 116/96[e] | Sokolov/Czech Republic | S | – | – | 1[a] |
| *Nostoc* sp. | Lukešová 6/99[e] | Germany | S | – | + | 54[a] |
| *Nostoc* sp. | Cam2S01[d] | Camerun | S | – | – | 48[a] |
| *Nostoc ellipsosporum* | Lukešová 51/91[e] | Nezamyslice/Czech Republic | S | + | – | 39[a] |
| *Nostoc commune* | NC1 | Třeboň/Czech Republic | S | – | – | 70 |
| *Nostoc commune* | NC2 | Třeboň/Czech Republic | S | – | – | 12 |
| *Nostoc commune* | NC3 | Nové Hrady/Czech Republic | S | – | + | 0 |
| *Nostoc commune* | NC4 | Nové Hrady/Czech Republic | S | – | – | 40 |
| *Nostoc commune* | NC 7 | České Budějovice/Czech Republic | S | + | n.a. | 34 |
| *Nostoc* sp. | NC 9 | Rožnov p. R.Czech Republic | S | – | n.a. | 27 |
| *Nostoc* sp. | OBU36S07[d] | The Burren Clare/Ireland | S | – | – | 42[a] |
| *Nostoc* sp. | CC2[d] | greenhouse Pisa/Italy | Sy | – | – | 30 |
| *Nostoc* sp. | CR4[d] | greenhouse Florence/Italy | Sy | – | – | 5[a] |
| *Nostoc* sp. | De1[d] | greenhouse Rome/Italy | Sy | – | – | 64[a] |
| *Nostoc* sp. | OGU 36S01[d] | Achill Island/Ireland | Sy | – | – | 31[a] |
| *Nostoc* sp. | Gm1[d] | greenhouse Siena/Italy | Sy | – | + | 14[a] |
| *Nostoc* sp. | Ds1[d] | greenhouse Rome/Italy | Sy | + | – | 54 |
| *Nostoc* sp. | Mm1[d] | greenhouse Rome/Italy | Sy | – | – | 0 |
| *A. mendotae* × *sigmoidea* | 04 06[c] | Březová/Czech Republic | Pl | – | – | n.a. |
| *Anabaena mendotae* × *sigmoidea* | 04 12[c] | Černiš/Czech Republic | Pl | – | – | 70 |
| *A. compacta* | 04 17[c] | Dubnenský/Czech Republic | Pl | – | – | 67 |
| *A. cf. curva* | 04 19[c] | Hejtman/Czech Republic | Pl | – | – | n.a. |
| *A. circinalis* × *crassa* | 04 22[c] | Husinec/Czech Republic | Pl | – | + | 50 |
| *A. lemmermannii* | 04 24[c] | Husinec/Czech Republic | Pl | – | – | 13 |
| *A. circinalis* × *crassa* | 04 26[c] | Jesenice/Czech Republic | Pl | – | + | 46 |
| *A. cf circinalis* | 04 28[c] | Hodějovický/Czech Republic | Pl | – | – | 29 |
| *A. lemmermannii* | 04 33[c] | Orlík/Czech Republic | Pl | – | – | n.a. |
| *A. lemmermannii* | 04 38[c] | Senecký/Czech Republic | Pl | – | – | 17 |
| *A. cf. flos-aquae* | 04 40a[c] | Skalka/Czech Republic | Pl | – | + | n.a. |
| *A. lemmermannii* | 04 42[c] | Svět/Czech Republic | Pl | – | + | 57 |
| *Aphanizomenon aphanizomenoides* | 04 43[c] | Svět/Czech Republic | Pl | – | + | 92 |
| *Anabaena affinis* | 04 44[c] | Svět/Czech Republic | Pl | – | + | 66 |
| *A. mendotae* × *sigmoidea* | 04 45[c] | Svět/Czech Republic | Pl | – | + | 87 |
| *A. cf. spiroides* | 04 51[c] | Svět/Czech Republic | Pl | – | + | 62 |
| *A. cf. flos-aquae* | 04 52a[c] | Svět/Czech Republic | Pl | – | + | 25 |
| *A. cf. flos-aquae* | 04 53[c] | Švarcenberk/Czech Republic | Pl | – | + | 53 |
| *A. circinalis* × *crassa* | 04 56[c] | Vajgar/Czech Republic | Pl | – | – | 47 |
| *A. cf. flos-aquae* | 04 57[c] | Vajgar/Czech Republic | Pl | – | + | 76 |
| *A. circinalis* × *crassa* | 04 59[c] | Valcha/Czech Republic | Pl | – | + | 82 |
| *Anabaenopsis cf. elenkinii* | Anaps Plást 05[c] | Plástovice/Czech Republic | Pl | – | + | n.a. |
| *A. compacta* | Acom Pěšák 06[c] | Pěšák/Czech Republic | Pl | – | + | 43 |
| *A. compacta* | Acom Svět 06[c] | Svět/Czech Republic | Pl | – | – | 2 |
| *A. lemmermannii – morfotypS* | Alem Lipno 05 silná[c] | Lipno/Czech Republic | Pl | – | – | 33 |
| *A. lemmermannii – morfotypT* | Alem Lipno 05 tenká[c] | Lipno/Czech Republic | Pl | – | + | 40 |
| *Anabaenopsis cf. elenkinii* | Anaps OLE-03[c] | Oleksovice/Czech Republic | Pl | + | + | 92 |
| *A. eucompacta* × *reniformis* | Anarenif Pěšák 2[c] | Pěšák/Czech Republic | Pl | – | + | 77 |

**Table 1** (*continued*).

| Scientific name | Strain | Place of isolation | Habitat | *A. salina* mortality | | Sp/2 inh. [%] |
|---|---|---|---|---|---|---|
| | | | | B | M | Mean |
| *A. eucompacta* × *reniformis* | Anarenif Pěšák 4[c] | Pěšák/Czech Republic | Pl | − | + | 90 |
| *Anabaena affinis* | Staňk 05-11[c] | Staňkovský/Czech Republic | Pl | − | + | 37 |

[a] Values of inhibition have been taken from Hrouzek et al. (2010).
[b] CCALA, the collection of phototrophic microorganisms, Institute of Botany, Třeboň, Czech Republic.
[c] The collection of the Insitute of Hydrobiology ASCR, České Budějovice, Czech Republic.
[d] The algal collection of the Institute of Ecosystem study, Florence (Italy).
[e] The collection of soil algae and cyanobacteria of the Institute of Soil Biology ASCR, České Budějovice, Czech Republic. Non marked strains are field samples.

trap. The extract was subjected to separation on a reversed phase column (Zorbax XBD C8, 4.6 × 150 mm, 5 μm) at 30 °C, and eluted by a gradient MeOH/$H_2O$ + 1% HCOOH (30–100% MeOH for 30 min, 100% for 5 min) with a flow rate of 0.6 mL $min^{-1}$. The obtained total ion chromatograms were evaluated and molecular ions were detected based on signal intensity, the presence of sodium and potassium adducts, and the distribution of isotopomeres.

### 2.4. Activity-guided fractionation

Six strains were selected for activity-guided fractionation in order to find out which fraction was responsible for the toxic effect. The strains *Cylindrospermum* sp. C24/1989, *Nostoc* sp. 6/99, *Nostoc muscorum* 14/86 and *Nostoc* sp. 5/96 were fractionated using an analytical column, gradient and conditions as discussed above (see also supplementary information). In strains *Nostoc ellipsosporum* 51/91 and *Nostoc* sp. Ds1 the fractionation was performed using preparative HPLC (LabAlliance, Watrex, Prague) on a reverse phase column (C18 Reprosil100, 250 × 8 mm, 5 μm, Dr. Maisch GmbH) and a modified gradient (see supplementary information). In these extracts the fractions were collected based on UV absorption in 237 and 220 nm for *N. ellipsosporum* 51/91 and *Nostoc* sp. Ds1, respectively. Finally, the collected fractions were evaporated and re-suspended to obtain the concentration equivalent to the original extract and tested for toxicity to *A. salina* and Sp/2 as mentioned above.

## 3. Results

A total of 63 cyanobacterial strains were included in the present study. The investigated cyanobacteria originated from four different habitats (plankton, soil, periphyton, and epiphytic communities), and besides these, strains originating from different symbiotic associations were also studied. For exact information about the origin and each strains' isolation see Table 1.

### 3.1. Toxicity of biomass extracts to A. salina

Fig. 1 shows the toxicities of the biomass extracts. An overall toxicity causing a mortality of *A. salina* higher than 50% was observed in 12.7% of biomass extracts. The highest occurrence of toxicity was found among strains originating from soil (22%). The most active soil isolates were *Nostoc* sp. 5/96 and *N. ellipsosporum* 51/91 that caused a 100% mortality, followed by *N. muscorum* 14/86 and *N. commune* NC7 (65% and 64.3% inhibition, respectively). In *Cylindrospermum* sp. C24/89, *Nostoc* sp. 116/96 and *Nostoc* sp. 6/99, a mortality slightly above 40% was found.

A lower toxicity occurrence of 14% was recorded for the symbiotic strains. Nevertheless, the symbiotic strain *Nostoc* sp. Ds1 exhibited a very strong and fast toxic effect manifested by the death of all animals within 24 h. Only one toxic strain was found in both epiphytic and periphytic cyanobacteria; however, the low number of tested strains from these habitats (four from either habitat) did not allow relevant conclusions to be made. The strain *Cylindrospermum* sp. Hrouzek 1/2004 isolated from leaves of water plants caused a strong toxic effect leading to a mortality of 100%. Out of 30 planktonic strains tested, only the strain *Anabaenopsis* cf. *elenkinii* Anaps OLE-03 caused a high mortality of *A. salina* (67%).

### 3.2. Toxicity of media extracts to A. salina

Fifty-eight media extracts were tested. 37.9% of all tested media extracts exhibited significant toxicity against *A. salina*. This high number can be attributed to the frequent occurrence of toxicity found in the media of planktonic cyanobacteria (Fig. 1). In contrast to the low incidence of biomass

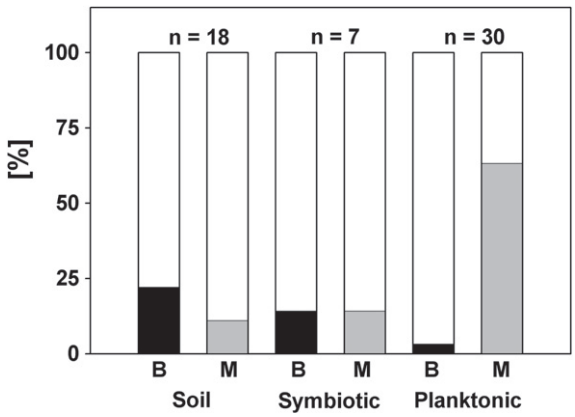


**Fig. 1.** Percentage of biomass (B) and medium (M) extracts causing lethal effects to *Artemia salina* in strains isolated from soil, different symbiotic associations and plankton. Black and grey parts of columns indicate toxicity occurrence in biomass and media respectively, white parts of columns correspond to percentage of non-toxic extracts.

toxicity in plankton strains, 63.3% of their media extracts exhibited a significant toxic effect. Among these, *Anabaena lemmermanii* (100% inhibition), *Anabaena cf. spiroides* 04 51 (91% inhibition) and *Anabaena circinalis/crassa* 04 22 (86% mortality) caused the strongest effect. The incidence of media toxicity was much lower in cyanobacteria from other habitats: 11.1% in soil strains and 14.3% in symbiotic strains. The medium extract of the symbiotic strain *Nostoc* sp. Gm1 caused significant mortality of *A. salina* (50% inhibition) in contrast to its biomass extract with no effect. On the other hand, the medium extract of the strain *Nostoc* sp. Ds1 lacked any effect in contrast to its biomass extract (see above). Soil strains *N. commune* NC2 and NC3 exhibited significant medium toxicity (46 and 50%, respectively) with no inhibition found in their biomass extracts. No toxicity of a medium extract was found in epiphytic strains and cyanobacteria isolated from periphyton.

### 3.3. Comparison of A. salina mortality with Sp/2 cell line inhibition values

No significant correlation between the toxicity of biomass extracts to Sp/2 cell line and to *A. salina* was found ($R = 0.1115$; $p = 0.4047$; Fig. 2). A high number of extracts (31% of all tested strains) were highly toxic to Sp/2 cell lines and non-toxic to *A. salina* (Fig. 2, area A), while only three strains were toxic to *A. salina* with no effect on the cell line (Fig. 2, area C). *Nostoc* sp. BR III exhibited strong toxicity to *Artemia* while having only a marginal effect on the cell line. In other strains belonging to area C (*N. commune* NC7 and *N. ellipsosporum* 51/91) a strong effect on *Artemia* was found; however, it was accompanied by a moderate cytotoxic effect to Sp/2. The activity of strains *Nostoc* sp. Mm1 and *Nostoc* sp. 116/96 (area B) to *Artemia* was apparent; however, toxicity was slightly under the artificial threshold value 50%. In these strains, no effect on the Sp/2 cell line was detected. The remaining strains, grouped in the area B of Fig. 2, had a low toxic effect to both *A. salina* and Sp/2 cell line.

Five of the tested strains (*Cylindrospermum* sp. Hrouzek 1/2004, *Anabaenopsis* cf. *elenkinii* Anaps OLE-03, *N. muscorum* 14/86, *Nostoc* sp. Ds1 and *Nostoc* sp. 5/96) were found to cause strong damage to both *Artemia* and cell lines (area D). In *N. ellipsosporum* 51/91, 6/99, *N. commune* NC7 and *Cylindrospermum* sp. C24/89, similar effects with inhibition values near the threshold value were found.

### 3.4. Fractionation of selected strains

Figs. 3 and 4 show chromatograms and the active fractions causing toxic effects to Sp/2 cell line and *A. salina* from extracts from the selected strains. Unfortunately, two other strains exhibiting the highest toxicity to both *A. salina* and Sp/2 cells (*Cylindrospermum* 1/2004 and *Anabaenopsis* cf. *elenkinii* Anaps OLE-03), could not be used due to problems with their cultivation and the low amount of extracts obtained.

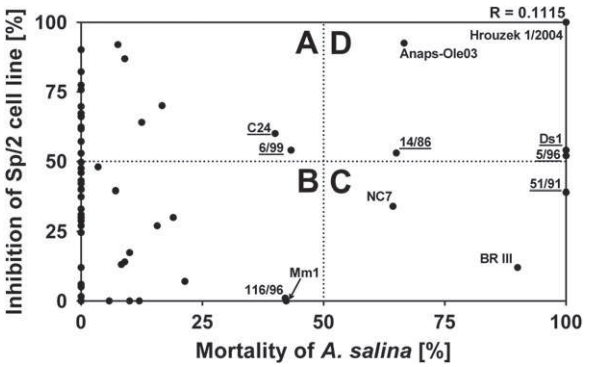


**Fig. 2.** Comparison of effects of biomass extracts of studied strains on *A. salina* mortality and Sp/2 cells inhibition. The non-significant correlation of inhibition values in *A. salina* and Sp/2 cells is obvious from the graph ($R = 0.1068$, $p = 0.4047$). The high number of strains toxic to Sp/2 cells with no activity to *A. salina* can be seen in Area B. By contrast, only a few extracts causing mortality to *A. salina* were not accompanied by a cytotoxic effect (Area C). The dotted lines represent the borders for both *A. salina* mortality and Sp/2 cell line inhibition values $\geq 50\%$. Extracts with an inhibition $\geq 50\%$ were considered strongly toxic. The names of considerably toxic strains are given. Underlined strains were selected for fractionation.

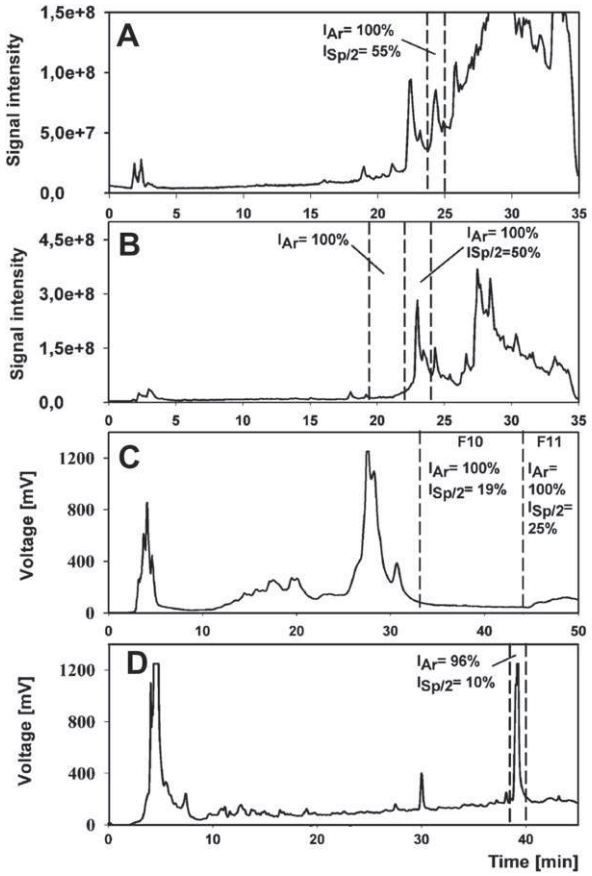


**Fig. 3.** Chromatograms of cyanobacterial strains in which only one fraction toxic to both *A. salina* and Sp/2 cells was observed. The active fraction is marked by dashed lines and the inhibition values of these fractions to *A. salina* ($I_{Ar}$) and Sp/2 cells ($I_{sp/2}$) are shown. A: *Cylindrospermum* sp. C24/89 (Total ion chromatogram), B: *Nostoc* sp. 5/96 (Total ion chromatogram), C: *Nostoc ellipsosporum* 51/91 (UV absorption at 237 nm), D: *Nostoc* sp. Ds1 (UV absorption at 220 nm).

In four of the six studied strains, the toxicity to *A. salina* and Sp/2 cell line was caused by an identical fraction or compound (Fig. 3). The toxicity of *Cylindrospermum* sp. C24/89 to both *A. salina* and Sp/2 cells was caused by a compound collected between 23.5′ and 25.0′ of analytical gradient. Within its mass spectrum, ions corresponding to molecular ion 1146 $[M+H]^+$ and its sodium adduct 1168 $[M+Na]^+$ were detected. The toxicity of this compound was also proved in its pure state obtained by preparative HPLC. In the strain *Nostoc* sp. 5/96, activity in both tests was caused by a compound with a molecular weight of 849.6, which exerted a 100% inhibition to both *A. salina* and Sp/2 cell lines. Additionally, fraction 3 was also highly toxic to *A. salina* with a mortality of 100% in an extract of this strain. In *N. ellipsosporum* 51/91 toxicity to the murine cell line and *A. salina* was caused by identical fractions. Fractions 10 and 11 with no UV absorption or clear peak in a total ion chromatogram were responsible for 100% mortality to *A. salina* in this strain and caused a moderate effect on the Sp/2 cell line (19% and 25% inhibition respectively). The strong toxic effect on *A. salina* was also observed for fraction 10 obtained from an extract of the strain *Nostoc* sp. Ds. The fraction was collected by preparative HPLC based on strong UV absorption at 220 nm and subsequent analysis proved the presence of a compound with a molecular weight of 460.1. The activity of this compound to Sp/2 cells was weak (10%); however, no other fractions of this extract exhibited an inhibition. Thus, it is possible that other components of the extract enhanced the inhibitory effect of this compound and the observed crude extract inhibition was therefore higher (54%).

Different compounds were responsible for the toxicity to *A. salina* and Sp/2 in the strains *N. muscorum* 14/86 and *Nostoc* sp. 6/99. In *N. muscorum* 14/86, fraction 3 containing a compound of MW = 885.0 (886 $[M+H]^+$; 908 $[M+Na]^+$) caused 61.1% mortality to *A. salina*, whereas 79% inhibition of the Sp/2 cell line was caused by fraction 9 containing a novel cyclic peptide of MW = 1211 (1212 $[M+H]^+$; 1234 $[M+Na]^+$) (Hrouzek et al., 2010). In the other strain *Nostoc* sp. 6/99, fractions 2 and 5 containing compounds of MW = 1006.9 and 1076.1, respectively, exhibited a significant lethal effect on *A. salina* (Fig. 4). The cytotoxicity of this strain was previously found to be caused by a fraction containing Nostopeptolide A1 (MW = 1081) (Golakoti et al., 2000; Hrouzek et al., 2010) and this result was confirmed by our study (Fig. 4).

## 4. Discussion

Our data demonstrate that 12.7% of the biomass extracts of all studied strains exerted a toxic effect on *A. salina*. This is in accordance with Jaki et al. (1999) and Mian et al. (2003), who found toxicity to *A. salina* in 13.9% and 9.1% of cyanobacterial strains, respectively. A slightly higher number of toxic strains (24%) were reported by Piccardi et al. (2000) who studied the bioactivities of *Nostoc* strains. Conversely, Falch et al. (1995) reported that 80% of all strains used in their study were toxic when tested on *A. salina*. However, the strains included in the study were selected for their previously reported pharmacological and biological effects, and so the frequency of the reported toxic strains therein could be misleading. Therefore, it seems

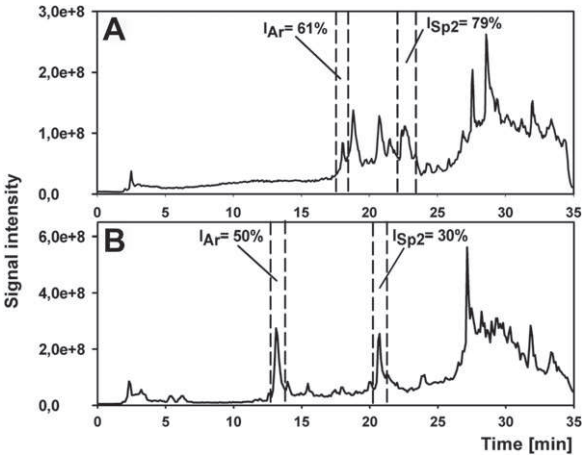


**Fig. 4.** Chromatograms of cyanobacterial strains in which distinct fractions were found to have a toxic effect on *A. salina* and Sp/2 cells. Active fractions are marked by dashed lines and inhibition values of these fractions to *A. salina* ($I_{Ar}$) and Sp/2 cells ($I_{sp/2}$) are shown. A: *Nostoc muscorum* 14/86 (Total ion chromatogram), B: *Nostoc* sp. 6/99 (Total ion chromatogram).

that the usual frequency of cyanobacterial strains toxic to *A. salina* ranges between 10 and 25%.

An *A. salina* toxicity assay has been suggested as a valid method to evaluate cytotoxic activity (Solis et al., 1993) and thus the method is commonly used as a substitute assay for the screening of cytotoxic compounds. However, Jaki et al. (1999), Mian et al. (2003), and Berry et al. (2004) found no correlation between *A. salina* mortality and cell lines inhibition values, and our data agree with their findings. Only 8.6% of the tested strains were toxic to both *A. salina* and the Sp/2 cell line. By contrast, 31% of the extracts exhibited a strong cytotoxic effect while did not cause any mortality to *A. salina*. Such frequency of cytotoxicity occurrence and lower number of extracts exhibiting positive toxic effect to *A. salina* indicates that in most cyanobacterial metabolites the toxic effect targets to various basal metabolic pathways present in the eukaryotic cell rather than being a specific mechanism against a complex multi-cellular organism. This result is strongly supported by the fractionation of extracts found to be toxic both to *A. salina* and Sp/2 cell lines. In four of the six fractionated extracts the toxic effects were caused by an identical compound, suggesting that the mechanism of the function is probably the same for both the cell and the complex organism. The activity to *A. salina* that was not accompanied by inhibition of Sp/2 cells was only found in the fractions of strains *N. muscorum* 14/86 and *Nostoc* sp. 6/99 and in three raw extracts (Mm1, BR III and 116/96). However, it is questionable whether the concentration of these compounds was sufficiently high to manifest the effect in the cells. Moreover, no particular compound toxic to *A. salina* was found in the strain *Nostoc* sp. BR III although the strain as a whole was found highly toxic to *Artemia;* the toxicity of this strain to Sp/2 cells was insignificant. So, the mechanism of toxicity of this extract to *A. salina* could be based on the synergic effect of many compounds present in the strain.

To conclude, the frequency of cyanobacterial extracts toxic to *A. salina* (12.7%) is much lower in comparison with occurrence of cytotoxicity, which was estimated to vary between 20 and 38% (Mian et al., 2003; Piccardi et al., 2000;

Surakka et al., 2005; Hrouzek et al., 2010). Only small portion of extracts (approx. 10% of extracts) were positive in both tests and in these the toxic effects were caused by identical compound for Sp/2 cells and *A. salina*. Based on these results we concluded that *A. salina* toxicity test is hardly applicable to estimate the possible health risk for human and in vitro mammal cells are much suitable for these purposes.

Finally, we tested if the differences in frequency of toxicity to *A. salina* existed among strains isolated from different habitats. The toxicity of biomass (intracellular) extracts was detected in 22% of the 18 soil strains. This is similar to the results of Jaki et al. (1999) who found toxicity in 16.6% of 30 studied soil strains. On the other hand, our results do not agree with data published by Mian et al. (2003) who did not found any toxic strain, and with data of Falch et al. (1995) and Piccardi et al. (2000), who observed toxicity in 83% and 75% of strains, respectively. However, Mian et al. (2003), Falch et al. (1995) and Piccardi et al. (2000) studied a low number (8, 12 and 8 resp.) of soil strains and thus reliable conclusions cannot be drawn. Only one out of 30 intracellular extracts (3.3%) of our planktonic strains exerted a toxic effect to *A. salina*. Although they used a lower number of strains, Mian et al. (2003) and Piccardi et al. (2000) published very similar results (0% of toxic strains in both). The occurrence of toxic strains among symbiotic cyanobacteria was over 14% in the present study, which is slightly lower compared to the results given by Piccardi et al. (2000), who observed toxicity in 6 out of 23 (26%) studied strains.

The distribution of toxicity to *A. salina* was different in cultivation media (extracellular compounds) between cyanobacteria originating from plankton and soil environment. The overall occurrence of toxicity in media extracts was lower than in the biomass of soil, subaerophytic, epiphytic and periphytic cyanobacteria. No toxic media extracts were found in epiphytic and periphytic strains and only a marginal occurrence of toxicity was observed in symbiotic and soil strains (14.3% and 11.1%, respectively). By contrast, a high occurrence of toxic media extracts was found among planktonic strains. However, only one toxic intracellular extract from planktonic strains was found as discussed above. A similar extracellular production of inhibitory compounds was published by Jüttner and Wessel (2003), who found that all five studied strains of *Cylindrospermum* synthesized and excluded zooplankton glucosidases' inhibitor DMDP – di(hydroxymethyl) dihydroxypirolidine. The major part of DMPD (80%) was found to be extracellular. Our data have revealed that planktonic cyanobacteria produce, and are able to release, compounds toxic to *A. salina*. More than 63% of media extracts of planktonic strains were found to be toxic to *A. salina*, whereas only one strain was found toxic among biomass extracts. In contrast, only 11.1% of media extracts of all soil strains were toxic to *A. salina*. These data are in agreement with the fact that in planktonic environment the diffusion of the toxin toward grazer is easy, while in the soil environment such production is not effective.

## 5. Conclusions

The present study has revealed that it is insufficient to monitor cytotoxicity of cyanobacteria by using only the *A. salina* bioassay as was usual in the past, since cytotoxicity is a more frequent feature in cyanobacteria in comparison with toxicity to *A. salina*. It seems that in most cyanobacterial metabolites the toxic effect targets various basal metabolic pathways present in the eukaryotic cell rather than being a specific mechanism against a complex multi-cellular organism. Therefore, we suggest that in vitro mammal cells be used when estimating the possible health risk for humans rather than *A. salina* toxicity test.

We have also revealed that there is a significant difference between the excretion of toxic extracellular metabolites between soil and planktonic strains. Exclusion of toxic metabolites into the surrounding environment (culturing media in our case) is far more frequent in plankton species than in soil species. In contrast, soil species produce toxic metabolites that are kept inside the cells. These data are in agreement with the fact that in plantktonic environment the diffusion of the toxin is easy, while in the soil environment such production is ineffective.

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## Appendix. Supplementary information

Separation gradients used for fractionation of the six selected strains. Standard separation gradient used for fractionation of extracts from strains *Cylindrospermum* sp. C24/1989, *Nostoc* sp. 5/96, *Nostoc muscorum* 14/86, *Nostoc* sp. 6/99. (A) Extracts from strains *Nostoc ellipsosporum* 51/91 and *Nostoc* sp. Ds1 was separated using gradients B and C, respectively. Percentage of methanol is marked by a solid line, water by a dashed line. Flow rate and monitored absorbance is shown in the lower left corner for B and C.

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.toxicon.2010.10.002.

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## Ethical statement

The authors hereby declare that this manuscript has never been published/submitted for the publication elsewhere. All authors have approved the final version of the article.

## Conflict of interest statement

There are no competing interests.

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