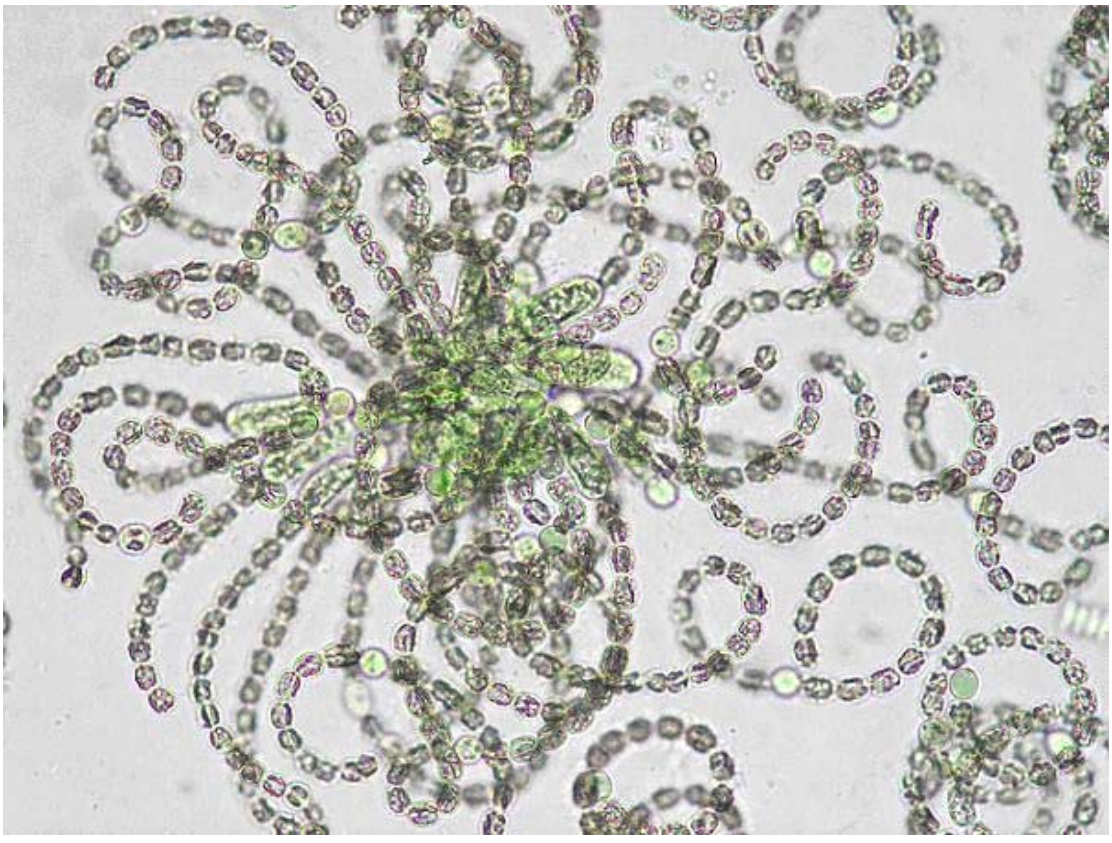
UNIVERSITY OF SOUTH BOHEMIA IN ČESKÉ BUDĚJOVICE
FACULTY OF SCIENCES

Bachelor Thesis

Daniel Hisem

Toxicity of Heterocyous Cyanobacteria to Model Invertebrate *Artemia salina*:

Is the toxicity specific and environmentally dependent?



Supervisor: Mgr. Pavel Hrouzek
Specialist: Ing. Jiří Kopecký, CSc.

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Annotation:

The aim of the study was to investigate toxicity of 65 heterocytous cyanobacterial strains originated from different habitats to *Artemia salina* and Sp/2 cell line. Extracts of toxic strains were analyzed by HPLC-MS to identify the composition. Active compound was targeted by activity-guided fractionation to find out toxin responsible for *Artemia* and Sp/2 cell line damage. Results of *A. salina* mortality were compared with Sp/2 cell line inhibition values. Finally, we concluded that cyanobacterial toxins are not primarily synthesized against grazers.

Anotace:

Cílem práce bylo stanovit toxicitu 65 kmenů heterocytózních sinic pocházejících z různých biotopů vůči modelovému bezobratlému organismu *Artemia salina* a savčí buněčné linii Sp/2. Složení extraktů s toxickým účinkem bylo stanoveno HPLC-MS analýzou. Za účelem zjištění zdali se na toxickém efektu k Artemiím a savčím buněčným liniím podílejí shodné látky byla provedena frakcionace s následným testem toxicity. Hodnoty mortalit *A. salina* byly porovnány s inhibicí Sp/2 buněčné linie. Na základě výsledků předpokládáme, že sekundární metabolity sinic nejsou syntetizovány primárně proti herbivorům.

I hereby declare that I worked up my bachelor thesis myself with assistance of cited literature and people mentioned in acknowledgement.

Prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění souhlasím se zveřejněním své bakalářské práce, a to v nezkrácené podobě, fakultou elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách.

Třeboň 4. 5. 2008 …….

Daniel Hisem

Poděkování

Děkuji všem, kteří smysluplně přispěli k dokončení této práce, čili svému kučeravému školiteli Pavlu Hrouzkovi a konzultantovi Jirkovi Kopeckému. Děkuji za veškerou pomoc Petru Tomkovi, Janičce Tomšic, Lucce Markové, Jítě za buchty, Ladě za udržování laborky a Pavlu Součkovi za cenné rady k počítačům. Všem dohromady chci moc poděkovat za příjemnou atmosféru v laborkách. Kejce děkuji za chvilky tělocviku při běhání po venku. Svým rodičům děkuji za veškerou hmotnou i duševní podporu v mém životě. Markétce děkuji za lásku a trpělivost, hlavně v posledních týdnech.

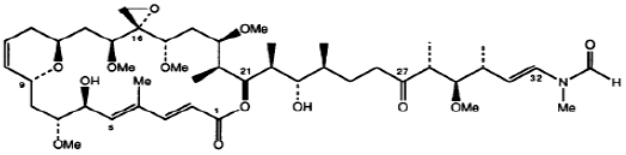
CONTENTS:

1. INTRODUCTION

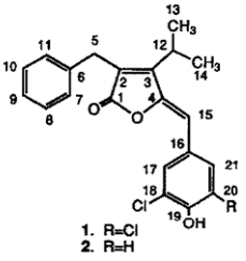
1. 1. The Cyanobacteria and their secondary metabolites

The cyanobacteria are remarkable group of autotrophic prokaryotic organisms which are well known as producers of a wide range of secondary metabolites that are not essential for the primary metabolism. In many cases, these secondary metabolites are proven to be toxic (e.g. Carmichael 1990b). Many of toxic cyanobacterial secondary metabolites have been already identified because of fast development of analytical methods. Cyanotoxins (as they are often called) are very diverse in biological activity and molecular structures. Based on biological activity, they are grouped into two main categories on **biotoxins** and **cytotoxins**. Evidently, biotoxins are able to kill multicellular organisms. On the other hand, cytotoxins are able to inhibit particular cells or unicellular organisms. Cytotoxins are further divided into several categories: hepatotoxins, neurotoxins, imunotoxins, genotoxins and embryotoxins (Maršálek 1996) based on affinity to cells of specific tissue. Effects are often combined (e.g. hepatotoxicity and nefrotoxicity). Of course, there is not "strict line" between the categories of bio- and cytotoxins, e.g. hepatotoxin is considered cytotoxic but can be biotoxic eventually.

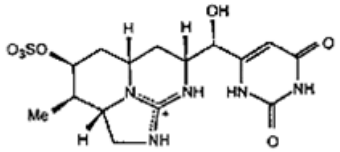
Cyanobacterial secondary metabolites are very heterogenous group from chemical point of view (Moore 1996, Burja et al. 2001). Production of cyclic and linear peptides, alkaloids, makrolide lactones, heterocyclic compounds and nucleoside derivatives has been reported from different cyanobacterial strains (Fig. 1).



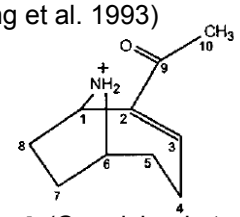
tolytoxin (Carmeli et al. 1993)



nostoclide I and II (Yang et al. 1993)



cylindrospermopsin (Harada et al. 1994)

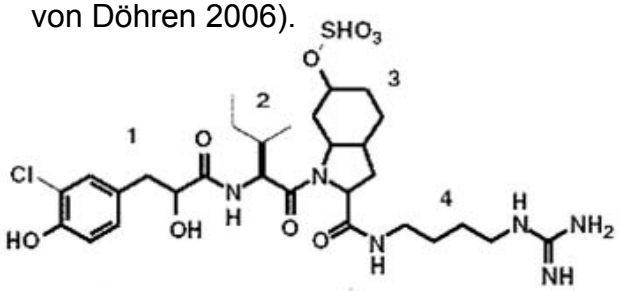


anatoxin A (Carmichael et al. 1979)

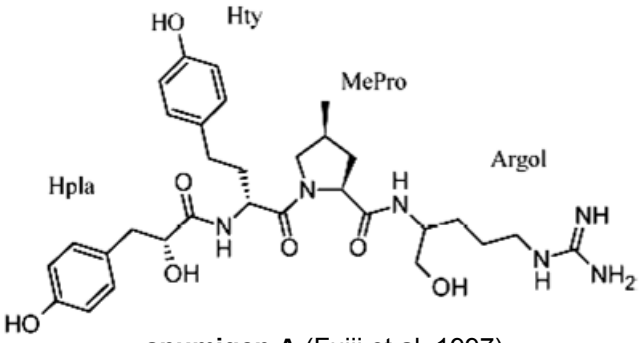
Fig. 1: Example of non-peptidical molecular structures of cyanotoxins.

From these, peptides are the most freqently found secondary metabolites produced in high concentrations. Cyanobacterial peptides consist of unusual amino-acids and are synthetized by the non-ribosomal synthetic patway (e.g. Welker et von Döhren 2006). This primitive biochemical proces is much older than the evolutionary history of the eucaryotic lineage (Rantala et al. 2003). So far, more than 600 peptides or peptidic metabolites have been described from various taxa. They are categorized into eight groups nowadays (Welker et von Döhren 2006).

1. aeruginosins – linear peptides characterized by a derivative of hydroxyl-phenyl acid (Hpla) at the N-terminus, the aminoacid 2-carboxy-6-hydroxyoctahydroindol (Choi) and an arginine derivative at the C-terminus (Murakami et al. 1995, Welker et von Döhren 2006).



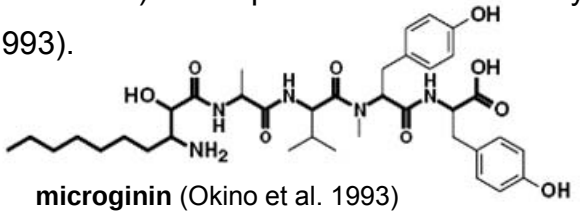
aeruginosin 98A (Murakami et al. 1995)



spumigen A (Fujii et al. 1997)

Fig. 2: Examples of molecular structures of aeruginosins.

2. microginins – linear peptides characterized by Adha (3-amino-2-hydroxy-decanoic acid) and a predominance of two tyrosine units at the C-terminus (Okino et al. 1993).



microginin (Okino et al. 1993)

cyanostatin A (Sano et al. 2005)

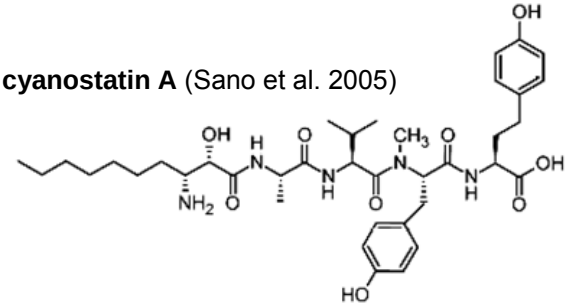
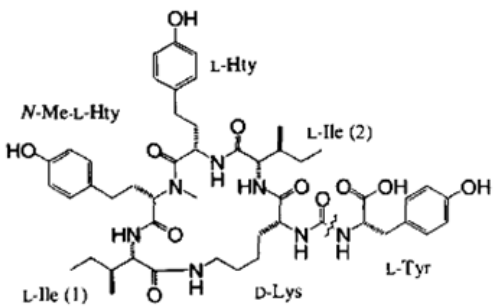
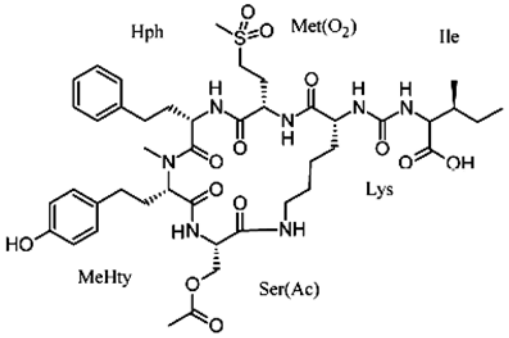


Fig. 3: Examples of molecular structures of microginins.

3. anabaenopeptins – cyclic peptides characterized by a lysine in position 5 and the formation of the ring by an *N*-*6*-peptide bond between lysine and the carboxy group of the amino acid in position 6. A side chain is attached to the ring.

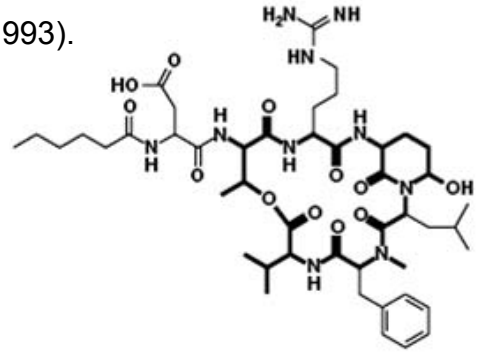


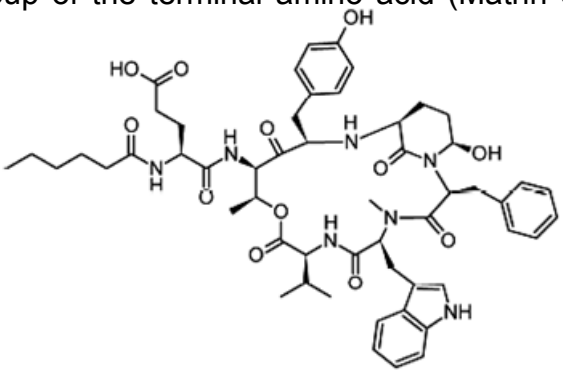


anabaenopeptin G (Itou et al. 1999) **nodulapeptin A** (Fuji et al. 1997)

Fig. 4: Examples of molecular structures of anabaenopeptins.

4. cyanopeptolins – cyclic peptides characterized by Ahp (3-amino-6-hydroxy-2-piperidone) and the cyclization of the peptide ring by an ester bond of the ß-hydroxy group of threonine with the carboxy group of the terminal amino acid (Matrin et al. 1993).

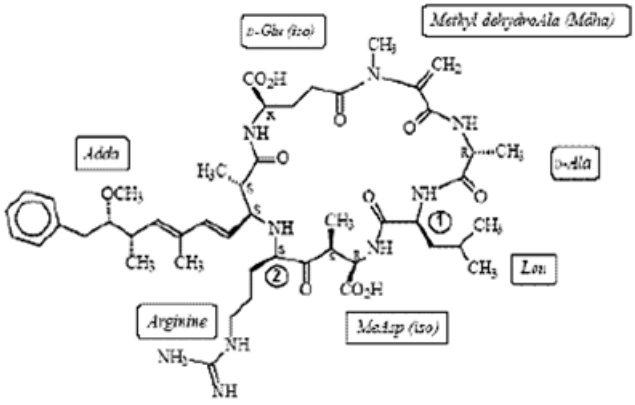




cyanopeptolin A (Martin et al. 1993) **micropeptin T1** (Kodani et al. 1999)

Fig. 5: Examples of molecular structures of cyanopeptolins.

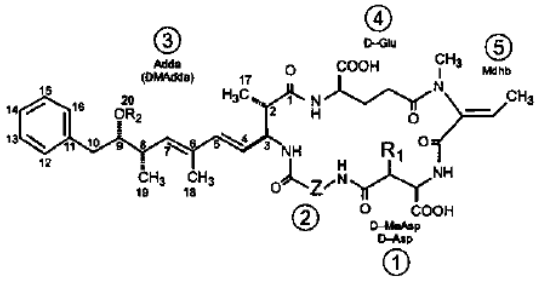
5. microcystins a nodularins – microcystins are cyclic heptapeptides with a common structure of cyclo-(D-Ala-L-**X**-D-*erythro*-ß-methylAsp(iso-linkage)-L-**Y**-Adda-D-Glu(iso-linkage)-N-methyldehydroAla) with variable X position (Arg, Leu, Tyr) and Y position (Ala, Arg, Tyr, Met) (Rinehart et al. 1988).



microcystin-LR (Harada et al. 1996)

Fig. 6: Example of molecular structure of microcystin-LR.

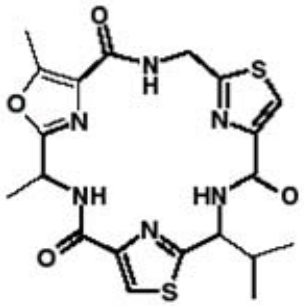
Structure of nodularins is highly similar to microcystin (cyclic, Adda), but are formed only by 5 amino acids.



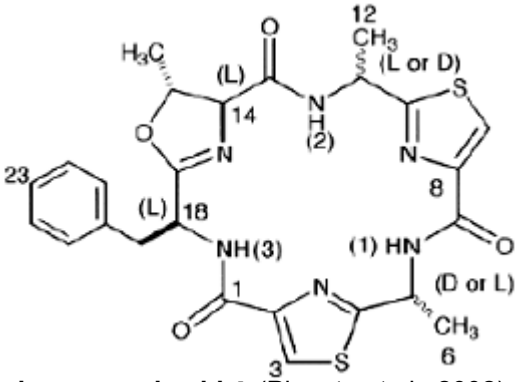
nodularin (Rinehart et al. 1988)

Fig. 7: Example of molecular structure of nodularin.

6. cyclamides – cyclic peptides with characteristic thiazole and oxazole moieties and 3 variable amino acids (Welker et von Döhren 2006 and references therein).



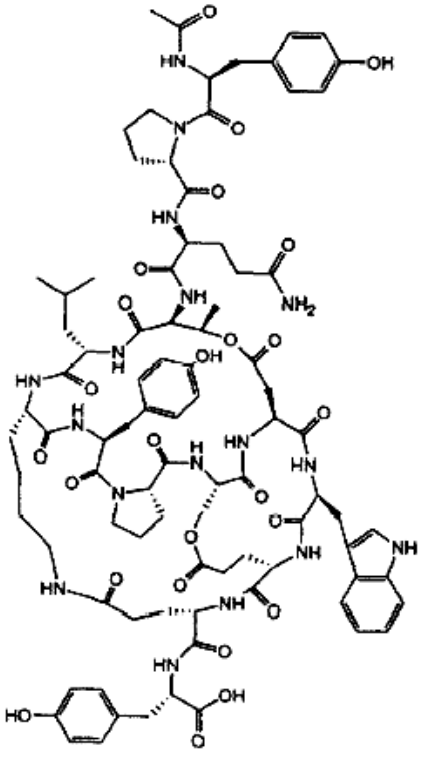
nostocyclamide (Todorova et al. 1995)



banyascyclamid A (Plounto et al., 2002)

Fig. 8: Examples of molecular structures of cyclamides

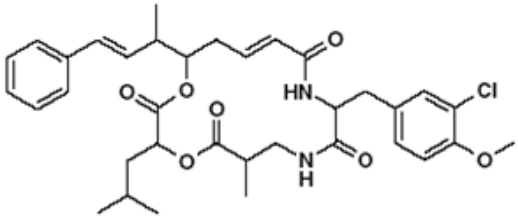
7. microviridins – the largest known cyanobacterial oligopeptides (14 amino acids). This group is characterized by a tricyclic structure established by secondary peptide and ester bonds and a side chain of a variable length (Ishitsuka et al. 1990).



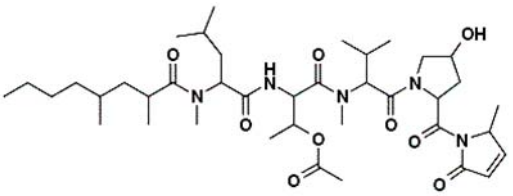
microviridin J (Murakami et al. 1997)

Fig. 9: Example of molecular structure of microviridins.

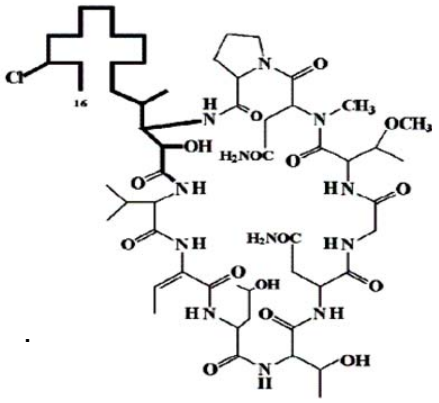
8. other peptides – remaining peptides cannot be grouped in larger classes with many structural variants. For most of these peptide types, only a few congeners are known and these often have been isolated as minor compounds from the same strain or sample (Welker et von Döhren 2006). Most of congeners of cyclic depsipeptides **cryptophycins** (Fig. 10) were isolated from a single strain of *Nostoc* sp. (Golakoti et al. 1994). Other minor group of peptides are e.g. **microcolins** (Koehn et al. 1992). Among other peptides I would like to mention e.g. puwainaphycins A-E (Fig. 10) isolated from *Anabaena* sp. (Gregson et al. 1992) or oscillatorin (Fig. 10) (Sano et Kaya 1996).



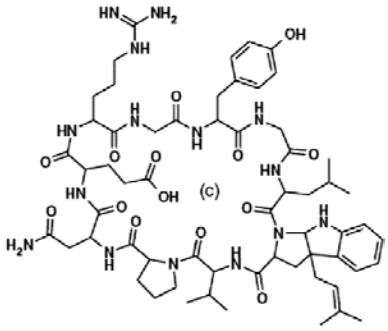
cryptophycin C (Golakoti et al., 1994)



microcolin A (Koehn et al., 1992)



puwainaphycin C (Moore et al. 1989)



oscillatorin (Sano et Kaya 1996)

Fig. 10: Examples of molecular structures of unclassified cyanobacterial peptides.

1. 2. Methods used in evaluation of toxicity to invertebrates

There are several methods (bioassays), which are commonly used for detection of toxicity of cyanobacterial strains extracts. There exists a wide range of the toxicity tests based on using different testing organism. Of these, we have to select the one that is optimal for our purpose.

The invertebrate based bioassays are successfully applied in many different ways of toxicity testing. These bioassays are simple and much cheaper compared to

any other methods. The standard *Artemia salina* bioassay is a useful screen for the toxicity-based detection of particular cyanotoxins (Metcalf 2002, Lincoln 1996). *A. salina* eggs are readily available from biological supply companies and can be stored for several years at -20 °C without loss of viability (Harada et al. 1999).

More invertebrate based toxicity bioassays were successfully applied by the time. The fairy shrimp, *Thamnocephalus platyurus* is known to be highly sensitive to cyanotoxins, especially to microcystins (Kozma 1997, Keil et al. 2002). Several species of *Daphnia* sp., (e. g. *D. galeata*, *D. pulicaria*, *D. magna* and *D. pulex*) are commonly used to detect cyanotoxins and to evaluate their effect on crustaceans (Rohrlack 1999, 2001, 2004; DeMott 1991). Also rotifer *Brachionus calicyfloris* has been tested for this purpose (Maršálek et Bláha 2000).

To expand the range of testing organisms, *Drosophila melanogaster, Moina macrocopa,* house flies or locust were also applied in toxicity testing, but have not been widely adopted (Swoboda et al. 1994, Agrawal 2005, Ross et al. 1985, McElhiney et al. 1998).

Of these invertebrate bioassays, the brine shrimp (*Artemia salina*) is most popular nowadays because no special equipment is required to maintain and handle the organism (Harada et al. 1999). *A. salina* bioassay is, as well as other assays mentioned above, commercially provided as a kit (ARTOXKIT-F, DAPHNOTOXKIT-F-magna, DAPHNOTOXKIT-F- pulex, THAMNOTOXKIT-F, ROTOXKIT-F). Although use of the kits increases the cost of performing the assay it brings high reproducibility and is time saving (24-h assays). Invertebrate bioassays are usually used to evaluate effects of particular compound, e. g. microcystins, cylindrospemopsin, pahayokolid (Metcalf et al. 2002, Berry et al. 2004), or to detect toxicity of crude extracts (e. g. Keil et al. 2002).

There are several levels at which the toxicity to invertebrates is studied and interpreted. Usually percentage mortality is determined. Crustacean proteases (trypsin, chymotripsin, cysteine, collagease) inhibition, which refers to lower digestivity of cyanobacterial biomass, can be evaluated. (Agrawal et al. 2005, Von Elert et al. 2004). Subsequent disruption of digestive system is also studied.

It is important to mention that spectrum of isolated compounds and subsequent acute toxicity often depends on used solvent and also on specific cell pre-treatment. Most used solvents are methanol, dichlomethane, hexane or water. Sonification of cyanobacterial cells results in higher toxicity. This method seems to be

more efficient in breaking down cyanobacterial cells than using mechanical forces (French pressing, grinding) (Keil et al. 2002). Keil et al. (2002) reported that acute toxicity of *Planktothrix* bloom to *T. platyurus* nauplii was more pronounced after sonification of cyanobacteria and subsequent extraction of metabolites by water, methanol or dichlormethane. Also increase of crustacean sensitivity to purified cyanobacterial extracts by manipulation of experimental conditions was investigated. It was found out, that exposure time, higher temperature and presence of DMSO (dimethylsulfoxide) can increase the sensitivity of microbiotests to microcystins (Drobniewska et al. 2004).

Since many scientists use different methods of toxicity testing, some studies compared sensitivity of particular methods. Maršálek et Bláha (2004) compared sensitivity of 17 acute bioassays of cyanobacterial toxicity by assesment of crude extracts of three cyanobacterial samples. Toxicity was tested on different organisms (see Table 1). The most sensitive bioassay was the 24-h test with crustacean *Thamnocephalus platyurus.*

group	representative
crustaceans	*Artemia salina (24-h, 48-h), Daphnia magna, D. pulex, Thamnocephalus platyurus, Ceriodaphnia dubia*
protozoans	*Tetrahymena pyriformis, T. thermophyla, Spirostomatum ambiguum*
rotifer	*Brachionus calicyfloris*
cnidaria	*Hydra tenua*
nematoda	*Panagrellus redivivus*
oligochaete	*Tubifex tubifex*
insect	*Culex pipiens, Drosophila melanogaster*
plant	*Sinapis alba, Lepidium sativum*

Table 1: Different organisms used most in acute toxicity testing (Maršálek et Bláha 2004).

1. 3. Effects of cyanobacteria on invertebrate grazers

To summarize the available literature dealing with effects of cyanobacteria on grazers I have decided to divide known studies into two groups. First group are **toxicological** studies, second are **ecological** studies. Toxicological studies are targeted on particular way of inhibition, in principle on chemical way of inhibition. Ecological studies describe changes in feeding and growth rate of grazers exposed to cyanobacteria. Mechanical interferences of grazers feeding apparatus with cyanobacterial morphology are also included.

1. 3. 1. Toxicological studies

Acute toxicity testing is the main method which can give us information about chemical effect of cyanobacteria on grazers. This method does not give us

information about mechanism of inhibition but mortality of testing organisms is estimated. We can determine concentration of toxin which is able to kill 50% of individuals (LC_{50}). Estimation of toxicity of crude extract is the first step in acute toxicity assays (Törökné et al. 2000, Keil et al. 2001).

Mechanisms of how cyanobacteria inhibit grazers by chemical interactions are investigated in laboratory experiments. Studies are targeted on inhibition of crustacean digestive enzymes, proteases mainly. The major digestive proteases of e.g. *Daphnia magna* have recently been characterized as trypsin and chymotrypsin (Von Elert et al. 2004), whereas the activity of cysteine protease was detected in the whole body homogenate of *Moina macrocopa* (Agrawal et al. 2005). Trypsin, chymotrypsin and collagenase are the common proteases characterized in higher crustaceans and the sequence data of proteases from a lower marine crustacean have been reported (Agrawal et al. 2005).

However, ecological implications of cyanobacterial protease inhibitors could not be addressed because mammalian rather than crustacean proteases have been used to demonstrate inhibitory effects. *Daphnia magna*, *Moina macrocopa* and *Thamnocephalus platyurus* have been used to demonstrate inhibition of crustacean proteases by cyanobacterial secondary matabolites. (Jakobi et al. 1996, Agrawal et al. 2005, Blom et al. 2003, Rohrlack et al. 2003).

Agrawal et al. (2005) examined the inhibition activity of *Microcystis aeruginosa* PCC7806 in experiments with the proteases of crustacean *Moina macrocopa*. Proteases present in the body of the crustacean are trypsin, chymotrypsin, elastase and cysteine protease. Their results showed that extract from *M. aeruginosa* exerted very strong inhibition of overall proteolysis of substrates suggesting that the metabolites present in this elute inhibit trypsin, chymotrypsin and cysteine protease activities. After further fractionation they obtain three fractions. F1 (fraction one) inhibited cysteine protease, F2 inhibited trypsin and F3, in addition to trypsin, inhibited chymotrypsin. No inhibitory effect was found for elastase. Interestingly, the single cyanobacterial strain contains inhibitors of all major proteases of the genus *Moina*. *M. aeruginosa* is known to produce cyclic peptides cyanopeptolins A-D. Cyanopeptolin A has been also shown to inhibit the bovine trypsin. Author suggested possibility that other cyanopeptoline like peptides are responsible for proteases inhibitory effects in *Moina* (Agrawal et al. 2005).

Microviridin J produced by *Microcystis aeruginosa* was determined as inhibitor

of daphnid trypsin (Rohrlack et al. 2004). It was shown in this study that almost all *Daphnia* individuals fed with the cyanobacteria were unable to swim and feed as a result of disability to shed the old integument which was attached to antenna and filter legs. Additionally, the entire body surface of *Daphnia* became rapidly covered with particles originating from the food suspension. The individuals had developed the molting disruption and died within the next few days.

In another study (Rohrlack et al. 2005) was demonstrated that production of inhibitors of daphnid trypsin is a common feature in the widely distributed *Planktothrix* strains of diferent species. About 70% of the 89 strains contained these potential *Daphnia*-toxins. However, the findings have also shown that not all *Planktothrix* strains synthesize inhibitors of daphnid trypsin. This emphasizes that the chemical ecology of cyanobacteria is determined at least partially by strain specific factors. It verifies the conclusions of earlier studies (e.g. Henning et al. 1991).

Also inhibitory effects to glucosidases have been recently found. Jüttner et Wessel (2003) investigated five strains of cyanobacterial genus *Cylindrospermum*. They found out that all five species synthesize DMDP – di (hydroxymethyl)dihydroxypyrolidine. The isolated compound inhibited digestive α- and ß-glucosidases isolated from crustacean zooplankton. DMDP is the first glucosidase inhibitor described in cyanobacteria. Interestingly, the major part of DMDP (80%) was found to be extracellular. Glucosidase inhibitors may favor the survival of cyanobacterial cells during passage through the digestive system of grazers because the glucosidase inhibitors help to maintain the integrity of the mucilage that shields the cells, as confirmed by experiments of Goarant et al. (1994). *Cylindrospermum* often forms biofilms that are exposed to grazing. So it is possible that *Cylindrospermum* produces the compound as the defense against grazing which is interesting ecological function, authors suggested.

Activity of artemid trypsin was already studied from different points of view (García-Ortega et al. 1998). However, inhibition of artemid trypsin by cyanobacterial secondary metabolites is not studied much.

Some studies have confirmed that invertebrates (e. g. *Artemia salina*) are able to fight against cyanobacterial toxins such as Microcystin LR, Microcystin HtyR and Nodularin via glutathione S-transferase GST detoxication (Beattie et al. 2003). All three toxins were conjugated via GST, which has been shown as an initial step of microcystin and nodularin detoxication. Yet, it was also found out that microcin S680

is able to inhibit the activity of both microsomal and soluble GST in *Daphnia magna* (Wiegand et al. 2002)

It is logical to suppose that there is no big difference between proteases inhibition and acute toxicity because it can be caused by proteases inhibition. But this question is not completely clear. Rohrlack (2004) in his work claimed that findings on the *Daphnia* toxicity of microviridin J link for the first time an acute toxic effect of cyanobacterial cells to a protease inhibitor contained within those cells. Finally, hundreds of other cyanobacterial metabolites with activities against proteolytic enzymes must be considered potentially toxic to zooplankton grazers.

Effects of particular cyanobacterial secondary metabolites have been studied recently. Rohrlack et al. (1999) studied role of **microcystins** in poisoning and food ingestion inhibition of *Daphnia galeata.* The study was based on comparative effects of microcystin-producing wild-type strain *M. aeruginosa* PCC7806 and mcy$^-$PCC7806 mutant, which could not synthesize microcystin due to mutation of a microcystin synthetase gene. The wild-type strain was found to be poisonous to *D. galeata* and the time needed to kill 50% of animals varied from 1.2 to 2.0 days. In contrast, mcy$^-$ mutant did not have any lethal effect on animals. But interestingly, both strains were able to reduce *Daphnia* ingestion rate. It was suggested that the poisoning of *Daphnia* and the inhibition of ingestion are caused by different factors. Furthermore, their results support the hypothesis that one function of microcystins could be to eliminate grazers of *Microcystis* spp. Basically the same results were published two years later again by Rohrlack et al. (2001).

Purified **cylindrospermopsin** and microcystin LR were studied by Metcalf et al. (2002). Cylindrospermopsin was slightly less toxic than microcystin-LR, with similar LC_{50} values on a gravimetric basis, but was more toxic to *A. salina* than the protein synthetases inhibitors, cycloheximide, chloramphenicol and tetracycline. Cylindrospermopsin-containing strains of the cyanobacterium *Cylindrospermopsis raciborskii* were found to be toxic to *A. salina* and the LC_{50} concentration for these strains over time was grater than the LC_{50} for purified cylindrospermopsin with the exception in one strain.

Curacin D, isolated from *Lyngbya majuscua,* was considered toxic to *A. salina* with LD_{50} = 40 ng/mL (lethal dose for 50 % of individuals) (Márquez et al. 1998). When tested against brine shrimp (*A. salina*), **pahayokolide A** was only marginally toxic at the highest concentrations (1 mg/ml) (Berry et al. 2004). The new type of

microcystin, **[D-Asp^3, (*E*)-Dhb^7]-microcystin-RR** was found most toxic to *Thamnocephalus platyurus* when compared with three known microcystins – microcystin-LR, -YR and –RR. In the same study, **nodularin** was found even more toxic than all microcystins (Blom et al. 2001). Also **Oscillapeptin J** isolated from the same *Planktothrix* strain was found to be a high potent crustacean grazer toxin when tested on *T. platyurus* (Blom et al. 2003).

Antillatoxin, **barbamide**, **dolastatin 3**, **G**, **11** and **12**, **grenadamide**, **hermitamide A–B**, **kalkitoxin**, **kalkipyrone**, **lynbyastatin 1** and **2**, **malyngamide A–U**, **oscillotoxin A**, **tanikolide**, **yanucamide A–B**, all isolated from *Lyngbya majuscula* from different localities were found to be toxic to *Artemia salina.* Six other compounds from *L. majuscula* did not affect *A. salina* (Burja et al. 2007). Data are summarized in the Table 2.

1. 3. 2. Ecological studies

Studies, which can be called “ecological”, are based on investigation of feeding rate, morphological interferences or growth rate measurements. In this chapter I focus mainly on consequences of cyanobacterial effects on grazers.

Cyanobacteria are often considered as a poor quality food for zooplankton. The low nutritional content of cyanobacteria, demonstrated as a low reproductive response, may be due to slow assimilation (Lampert 1985) or lack of essential compounds (Holm 1984; Brett 1997; Müller-Navarra 2000) such as highly unsaturated fatty acids - HUFA (DeMott 1997). Saturated fatty acids (SAFA) are important due to their high calorific content and are mainly utilized for energy, whereas polyunsaturated fatty acids (PUFA) affect the production of eicosanoids, which are crucial for physiological function related to reproduction in invertebrates, e.g. egg production and hatching (Brett 1997 and references therein). However, lack of essential compounds in cyanobacteria is not common feature. Nagarkar (2004) confirmed that cyanobacterial biofilms from Hong Kong rocky shores showed high nutritional quality in terms of protein, carbohydrate and calorific value.

Nevertheless, there are other reasons for considering cyanobacteria as poor quality food for grazers. As mentioned in previous chapter, cyanobacterial toxins may have severe consequences for grazers. Table 3 shows list of cyanobacterial strains found to be toxic to invertebrates. These invertebrates are commonly used for toxicity assays but are not always living in the same habitat with cyanobacterium and such

data are poorly useful for ecological estimation. However, they give us good information about secondary metabolites present in the cyanobacteria and their general toxic effects.

Toxin	Testing organism	Toxic effect/concentration	Exposition time	Reference
cylindrospermopsin	*Artemia salina*	LC_{50} - 4.48 mg/ml	24 h	Metcalf et al. 2002
		LC_{50} - 2.94 mg/ml	40 h	
		LC_{50} - 2.86 mg/ml	48 h	
		LC_{50} - 2.38 mg/ml	64 h	
		LC_{50} - 0.71 mg/ml	72 h	
mycrocystin-LR	*Artemia salina*	LC_{50} - 4.58 mg/ml	24 h	Metcalf et al. 2002
		LC_{50} - 3.00 mg/ml	40 h	
		LC_{50} - 2.80 mg/ml	48 h	
		LC_{50} - 1.19 mg/ml	64 h	
		LC_{50} - 0.85 mg/ml	72 h	
microcystins	*Artemia salina*	LC_{50} - 2.476 - 9.269 mg/ml varied between	24 h	Dronniewska et al. 2004
microcystins	*Thamnocephalus platyurus*	LC_{50} - 0.819 - 2.495 mg/ml >iffer in defferent conditions	24 h	
type of microcystin-RR		LC_{50} - 3.6 µM		Blom, J. F. et al. 2001
microcystin-LR		LC_{50} - 8.6 µM		
microcystin-YR	*Thamnocephalus platyurus*	LC_{50} - 6.1 µM	24 h	
microcystin-RR		LC_{50} - 8.3 µM		
nodularin		LC_{50} - 1.4 µM		
microcystin-LR	*Artemia salina*	EC_{50} - 3.7 mg/ml [a]	24 h	Maršálek et Bláha 2004
		EC_{50} - 2.2 mg/ml [a]	48 h	
	Daphnia magna	EC_{50} - 5.5 mg/ml [a]	48 h	
	Daphnia pulex	EC_{50} - 1.1 mg/ml [a]	24 h	
	Ceriodaphnia dubia	EC_{50} - 6.1 mg/ml [a]	24 h	
	Thamnocephalus platyurus	EC_{50} - 0.31 mg/ml [a]	24 h	
	Brachyonus caliciflorus	EC_{50} - 14.1 mg/ml [a]	24 h	
microcystins	*Daphnia similis*	EC_{50} - 46.00 µg.g^{-1} [b]		Okumura et al. 2007
		EC_{50} - 34.20 µg.g-1 [b]	48 h	
		EC_{50} - 1.38 µg.g-1 [b]		
microcystins	*Ceriodaphnia dubia*	EC_{50} - 73.1 µg.g-1 [b]		Okumura et al. 2007
		EC_{50} - 32.6 µg.g-1 [b]	48 h	
		EC_{50} - 1.470 µg.g-1 [b]		
microcystins	*Ceriodaphnia silvestrii*	EC_{50} - 80.2 µg.g-1 [b]		Okumura et al. 2007
		EC_{50} - 35.8 µg.g-1 [b]	48 h	
		EC_{50} - 1.44 µg.g-1 [b]		
microcystins	*Ceriodaphnia silvestrii*	C (µg.g^{-1}) 7.8 15.5 31.1		Okumura et al. 2007
		mortality % 20.0 50.0 80.0	48 h	
pahayacolide A	*Artemia salina*	max. 55% mortality at c = 1 mg/ml	24 h	Berry et al. 2004
curacin D	*Artemia salina*	LD_{50} - 40 ng/ml	24 h	Márquez et al. 1998
oscillapeptin J	*Thamnocephalus platyurus*	LC_{50} - 15.6 µM	24 h	Blom, J.F. Et al. 2003
cryptophycin	*Artemia salina*	toxic at c = 0.27 mg/ml	24 h	Biondi et al. 2004
microcin S680	*Daphnia magna*	inhibition of m- α s-GST	24 h	Wiegand et al. 2002
microviridin J	*Daphnia pulicaria*	molting disruption in 50% of ind. at concentration of 12 mg/l = 1.5 days	1.5 days	Rohrlack et al. 2004
microviridin J	*Daphnia pulicaria*	100% mortaity in 5 days	-	Rohrlack et al. 2004
		50% mortality in 3.7 days	-	
microviridin J	*Daphnia pulicaria*	conc. of 4.5 mg/l toxic for 50%	-	Rohrlack et al. 2004
		conc. of 6.75 mg/l toxic for 50%	2.6 days	
		conc. of 12 mg/l toxic for 50%	1.6 days	
DMDP* (major part in media)	*Crustacean zooplankton*	IC_{50} for &- and ß-glucosidases 19 and 49 nM respectively		Jüttner et Wessel 2003
	Thamnocephalus platyurus	not toxic up to 100 µM	24 h	

[a] EC_{50} mg biomass d.w./ml
[b] EC_{50} (µg microcystin/g dry weight of freeze-dried cells)
*di (hydroxymethyl)dihydroxypyrolidine
LC_{50} - lethal concentration for 50% of individuals
EC_{50} - effective concentration for 50% of individuals
IC_{50} - inhibitive concentration for 50% of individuals
LD_{50} - lethal dose for 50% of individuals

Table 2: Cyanotoxins found to be toxic to invertebrate models.

Morphology and mucilage of cyanobacteria has to be also included as factor influencing grazing on cyanobacteria. Cyanobacteria with filamentous or colonial morphology form aggregates that could reduce feeding rates or clog the feeding apparatus of grazer (Webster 1978). Wilson (2006) synthesized data from 66 published laboratory studies to get the information about cyanobacteria - grazer interactions. He concluded that diets containing filamentous cyanobacteria are less inhibitory to grazers than diets containing single-celled and colonial cyanobacteria. Moreover, it was revealed that filamentous cyanobacteria are significantly better food for grazers than single celled cyanobacteria (Wilson 2006). However, it was concluded that chemical defenses of cyanobacteria against grazing are more important than morphological features (Kurmayer et Jüttner 1999). DeMott (1986) suggested that also bad taste factor or bad odour is characteristic of poor food quality of cyanobacteria.

Cyanobacterium found to be toxic to invertebrate	Invertebrate	Reference
Nodularia harveyana	TP, BC	Pushparaj et al. 1999
Planktothrix aghradii	TP, DM	Keil et al. 2002; Törökné et al., 2000; Rohrlack et al. 2005
Planktothrix rubescens	TP, DM	
Planktothrix pseudaghadrii	DM	
Microcystis PCC7813	AS	Metcalf et al., 2002
Cylindrospermopsis raciborskii	AS	
Microcystis ichtyoblabe	AS	Sabour et al., 2001
Microcysitis aeruginosa PCC7806	DG, MM	Rohrlack et al., 1999; Agrawal et al. 2005
Microcysstis aeruginosa	TP	Törökné et al., 2000
Anabaena 66A	TP	
Anabaena 66B	TP	
Anabaena 83/1	TP	
Anabaena 123	TP	
Hydrocoleus sp.	AS	Mian et al. 2003
Fischerela ambigua	AS	Jaki et al. 1999
Lyngbya sp.	AS	
Nostoc commune	AS	
Scytonema myochrous	AS	
Toplypothrix bissoidea	AS	
Aphanizomenon flos-aquae	AS	Falch et al. 1995
Nostoc phaericum	AS	
Scytonema lynbyoides	AS	
Scytonema myochrous	AS	
Phormidium autumnale	AS	
Tolypothrix distorta var. *symplocoides*	AS	
Cylindrospermum licheniforme	TP	Jüttner et Wessel 2003
Cylindrospermum sp.	TP	
Trichodesmium thiebautii	AS	Hawser et al. 1992
Oscilatoria coraciana	AS	Smith 1996
Nostoc ATCC 53789	AS	Biondi et al. 2004
Synechococcus sp.	AS	Martins et al. 2007
Synechocystis sp.	AS	

Table 3: Cyanobacteria found to be toxic to invertebrates (TP – *Thamnocephalus platurus*, BC – *Brachionus calicyfloris*, DM – *Daphnia magna*, DG – *Daphnia galeata*, AS – *Artemia salina*, MM – *Moina macrocopa*).

According to Lürling (2006), addition of *Mycrocystis aeruginosa* into the diet severely depressed growth and reproduction in *Daphnia* and posses a severe threat to its survival. (Only a switch in reproductive strategy might provide *Daphnia* a refuge to a *Microcystis* environment that gradually becomes uninhabitable). Six *Daphnia* clones (*D. galeata* A and B, *D. hyalina, D. pulicaria, D. pulex, D. magna*) were studied by Rohrlack (2001), who compared effects of the microcystin-producing *Microcystis* strain PCC7806 and its mutant, which has been genetically engineered to knock out the microcystin synthesis. Microcystins produce by the *Microcystis* cells were poisonous to all *Daphnia* clones tested and animals died significantly faster than the animals fed with microcystin-lacking mutant. Despite it, both variants of PCC7806 were ingested at low rates and thus it was suggested that Daphnia are not able to distinguish between microcystin-producing and -lacking cells (Rohrlack 2001). On the other hand, Carlsson (1995) hypothesized that a herbivore can distinguish a toxic cell, either by recognizing the toxin prior to ingestion of the cell, which would indicate the presence of an extracellular toxin in the water, or by “learning”, which would indicate the prior ingestion of a toxic cell, and subsequent avoidance due to its unpleasant taste or odour.

Surprisingly, it was found out that some grazers adopt ability to tolerate presence of cyanobacteria. Growth rates of *Daphnia* clones isolated from high-nutrient lakes (based on phosphorus concentration) were higher, and showed less relative inhibition on the cyanobacterial diet compared to clones isolated from low-nutrient lakes. Cyanobacteria are well known to be more prevalent at high total phosphorus (TP) concentrations than at low TP. Therefore it was suggested that *D. pulicaria* population exposed to high cyanobacterial abundance over long periods of time can adopt tolerance to toxic cyanobacteria in the diet (Sarnelle et Wilson 2005). Gilbert (1990) found that a toxic strain of *Anabaena* reduced the growth rate of one clone of *Daphnia* to near zero while having no effect on another clone. Another study suggests that *D. pulex* is more sensitive than *D. pulicaria* to inhibition by cyanobacteria (DeMott 1991).

Complexity of the interaction between cyanobacteria and grazer is evident from literature cited above. Wilson and Hay (2007) have revealed recently that some *Daphnia* strains are and some strains are not harmed by the consumption of microcystin-LR. The *Daphnia* that performed better on a diet containing live *Microcystis aeruginosa* showed reduced population growth when exposed to

microcystin-LR-treated *Chlorella* diet, whereas the *Daphnia* that performed poorly on the diet containing live *Microcystis* was not affected by the experimental diet containing microcystin-LR.

There are different interpretation on the function of microcystin and its advantage for the cyanobacteria. Some authors concluded that microcystins play a role in the defense of *M. aeruginosa* cells against zooplankton grazing (Kurmayer et Jüttner 1999). The results obtained, however, are inconsistent (Rohrlack 1999) because e.g. daphnids were able to feed on microcystin-containing *M. aeruginosa* without suffering any harmful effects (Matveev et al. 1994).

As it is clear from the study of Rohrlack et al. (2005), toxicity to *Daphnia* is common feature among planktonic strains of *Plakntothrix.* Of the 89 strains studied, about 70% were toxic to *Daphnia* and produced inhibitors of daphnid trypsin. Nevertheless, Rohrlack studied only planktonic species without comparison to cyanobacteria from different habitats. Piccardi et al. (2000) studied fifty cyanobacterial strains from different habitats (symbioses, soil, fresh and marine waters) belonging to the genus *Nostoc*. Surprisingly, there was a high number of symbiotic strains toxic to *A. salina* but no planktonic strain was found to be toxic. According to Falch et al. (1995), 15 out of the 20 investigated strains were toxic to *A. salina*. Most toxic were soil, subaerophytic and planktonic strains. High number of toxicity within the subaerophytic strain is supported also by Jaki et al. (1999). Data from above cited studies are summed in the Table 4.

Total No. of strains tested	No. of toxic strains	testing organism	No. of toxic strains													References
			soil		subaerophyt		planktonic		symbiotic		epiphytic		periphyton		other	
43	6 (13.9%)	*A. salina*	0 (4)	0%	5 (26)	19%	-	-	-	-	-	-	1 (11)	9%	-	Jaki et al. 1999
22	2 (9.1%)	*A. salina*	-	-	0 (8)	0%	0 (3)	0%	0	0%	-	-	0 (1)	0%	2	Mian et al. 2003
89	62 (70%)	*D. Magna*	-	-	-	-	62 (89)	70%	-	-	-	-	-	-	-	Rohrlack et al. 2005
50	12 (24%)	*A. salina*	4 (5)	80%	2 (3)	67%	0 (7)	0%	6 (23)	26%	-	-	-	-	-	Piccardi et al. 2000
20	15 (75%)	*A. salina*	4 (5)	80%	6 (7)	86%	2 (3)	67%	1 (1)	100%	1 (1)	100%	1 (2)	50%	-	Falch et al. 1995
Σ 224	95 (42.4%)		8	53%	13	43%	64	34%	7	42%	1	100%	2	20%		

Table 4: Summary of studies investigating habitat dependent toxicity of cyanobacteria to invertebrates.

1. 4. Specificity of cyanobacterial toxicity to model invertebrate *Artemia salina*

The *Artemia salina* toxicity assay has been suggested as a valid method to evaluate the cytotoxic activity of plant extracts (Solis et al. 1993) and as a rapid preliminary screening for toxic cyanobacteria (Lahti et al. 1995). The assay is based on the premise that bioactive compounds are often toxic in high doses and that *in vivo* lethality in a simple organism can be used as a convenient monitor for screening and

fractionation in the discovery of new cytotoxic natural products (McLaughlin et al. 1991). Some published data suggest a good correlation between the activity in the brine shrimp assay and the cytotoxicity against some tumor cell lines (Anderson et al. 1991) as well as hepatotoxic activity (KIVIRANTA et al. 1991). The assay is therefore usually used as a low cost and easily achievable cytotoxicity test replacing cell lines assays.

However, there are several studies from last years that present opposite results. A total of 86 lipophilic and hydrophilic extracts obtained from 43 cyanobacterial samples have been screened for their biological activities by JAKI et al. (1999). Extracts were tested on *Artemia salina*, KB cells (human nasopharyngal carcinoma) and Caco-2 cells (human colon adenocarcinoma). A lethal effect (lethality » 60%) against brine shrimp was exhibited by 8.1% of all extracts. Cytotoxic activity against KB cells was shown by 1.2%, and 8.1% were active against Caco-2 cells. There was no correlation between brine shrimp lethality and cytotoxicity against KB cells and only two extracts were active against brine shrimp and Caco-2 cells simultaneously.

MIAN et al. (2003) investigated 44 extracts from 22 cyanobacterial samples by similar method as JAKI et al. (1999). Only two extracts exhibited a significant activity (lethality »50 %) against *Artemia salina* but 38.6% exttracts were cytotoxic to KB cells. There was no correlation observable between cytotoxicity against KB cells and brine shrimp lethality again Also Berry et al. (2004) concluded similar results. Regarding these results, it may not be possible to monitor cytotoxicity using only the brine shrimp bioassay rather than cytocoxicity assay. Nevertheless, *Artemia salina* bioassay is still considered a good method for investigation of acute toxicity.

1. 5. Aims of my study

- Determination of toxicity of crude cyanobacterial strains extracts originated from different habitats to model invertebrate *Artemia salina*.
- Identification of active compound for toxic strains.
- HPLC-MS analysis (High performance liquid chromatography-mass spectrometry analysis) of active extracts.
- Comparison of occurrence frequency of toxic compounds in cyanobacteria originated from different habitats.
- Comparison of the toxicity of extracts to *A. salina* with the inhibition of mammal cell lines in order to find out whether the toxicity is caused by the same compound.

2. MATERIAL AND METHODS

2. 1. Origin and cultivation of cyanobacterial strains

Total number of 65 different cyanobacterial strains was taken into this study. 23 cultured strains and 7 field samples belonging to genus *Nostoc,* 2 strains of the genus *Cylindrospermum*, one strain of the genus *Calothrix* and *Trichormus*, 30 isolates of the genus *Anabaena* and one isolate of unknown cyanobacterium belonging to family *Rivulariaceae*. Strains originate from the collection of Ing. Alena Lukešová (Institute of Soil Biology – CAS), Dr. Stefano Ventura (Consiglio Nationale Richereche, Institute for Ecosystem studies, Florence) and from the unofficial collection of the Institute of Microbiology, Department of Autotrophic microorganisms, CAS, Třeboň. Planktonic species of the genus *Anabaena* and family *Rivulariacae* have been kindly provided and cultivated by MSc. Eliška Zapomělová (Institute of Hydrobiology – ASCR, České Budějovice).

Strains originate from different habitats (soil, planktonic, symbiotic, subaerophytic and epiphytic – Table 4). Almost all strains were cultivated in Allen and Arnold medium (Arnon 1974) in 300 ml cylindrical flasks, sparged with 2% CO_2 and illuminated with artificial light of the intensity of 70 W/m^2 (OSRAM DULUX Philips L) for 2-4 weeks. Planktonic species of the genus *Anabaena* were cultivated in the WC medium (Guillard et Lorentzen 1972) in 250 ml Erlenmeyer's flasks, illuminated with artificial light of the intensity of 50 $\mu mol.m^{-2}.s^{-1}$ for 3-4 weeks.

2. 2. Biomass and medium harversting and preparation of extracts

Biomass was harvested by centrifugation in 50 ml glass cuvettes (4500rpm, 15 min.), stored at -80°C and lyophilized. Cultivation medium was separated from biomass after centrifugation, 50 ml were filtrated using bacteriological filters and water-pump to obtain cell free medium.

200 mg of lyophilized biomass was extracted into 10 ml of 70% methanol (MeOH) in test-tubes for 1 h on dark place. Test tubes were centrifuged (4500 rpm, 15 min.), supernatant was transferred and evaporated by vacuous drier. Solid extract was resuspended in 1 ml of 70% MeOH to get the extract of concentration 200 mg of dry weight/ml.

To obtain extract from medium, 50 ml of filtrated medium was concentrated into 2 ml 100% methanol using solid phase extraction (MCX Cartridge OASIS, Waters) and vacuum-pump (SUPELCO, Visiprep).

scientific name	strain	year of izolation	locality	habitat
Nostoc sp.	**BR III**	2004	Paranapiacaba/Brazil	epiphytic (rain forest)
Nostoc sp.	**RQ2**	2004	Paranapiacaba/Brazil	epiphytic (rain forest)
Nostoc sp.	**Nostoc II B**	2004	Paranapiacaba/Brazil	epiphytic (rain forest)
Nostoc sp.	**NO Bromel**	2004	Paranapiacaba/Brazil	epiphytic (rain forest)
Nostoc sp.	**LC17S01**	2001	Alberta/Canada	lake periphyton
Nostoc sp.	**OSNI 32S01**	2000	Sítinový pond/Czech Republic	periphyton
Cylindrospermum sp.	**Cy OM**	2003	Zliv/Czech Republic	periphyton
Calothrix sp.	**HROUZ 2/2005**	2005	San Monoron-BUSRA/Cambodia	periphyton
Nostoc calcicola	**NMB 17**	1989	Havana/Cuba	soil/humid forest
Nostoc sp.	**NMB 9**	1996	Sokolov/Czech republic	soil
Nostoc muscorum	**N. Muscorum I**	1991	Nezamyslice/Czech republic	soil
Cylindrospermum sp.	**C 24**	1995	Ellesmere island/Canada	soil
Trichormus variabilis	**ISB 13**	1988	Dlouhá Ves/Czech Republic	soil (agricultural field)
Nostoc sp.	**NMB 27**	1999	Germany	soil (dump)
Nostoc sp.	**NMB 28**	1997	Germany	soil (dump)
Nostoc sp.	**N 8**	1996	Sokolov/Czech republic	soil (dump)
Nostoc sp.	**NMB 26**	1999	Germany	soil (dump)
Nostoc sp.	**Cam 2S01**	2006	Camerun	soil (ricefield)
Nostoc ellipsosporum	**N.ell**	1991	Nezamyslice/Czech republic	soil
Nostoc commune	**NC1**	2006	Třeboň/Czech republic	soil (subaerophyt)
Nostoc commune	**NC2**	2006	Třeboň/Czech republic	soil (subaerophyt)
Nostoc commune	**NC3**	2006	Nové Hrady/Czech republic	soil (subaerophyt)
Nostoc comunne	**NC4**	2006	Nové Hrady/Czech republic	soil (subaerophyt)
Nostoc commune	**NC 10**	2006	unknown	artifitial substrata
Nostoc commune	**NC 7**	2006	Ćeské Budějovice/Czech republic	artifitial substrata
Nostoc sp.	**NC 9**	2006	Rožnov p. R.Czech republic	artifitial substrata
Nostoc sp.	**OBU36S07**	2000	The Burren Clare/Ireland	subaerophytic
Nostoc sp.	**CC 2**	1992	greenhouse Pisa/Italy	symbiont (*Cycas circinalis*)
Nostoc sp.	**CR 4**	1992	greenhouse Florence/Italy	symbiont (*Cycas revoluta*)
Nostoc sp.	**De1**	1992	greenhouse Rome/Italy	symbiont (*Dioon edule*)
Nostoc sp.	**OGU 36S01**	2000	Achill Island/Ireland	symbiont (*Gunerra manicata*)
Nostoc sp.	**Gm1**	1992	greenhouse Siena/Italy	symbiont (*Gunerra manicata*)
Nostoc sp.	**Ds1**	1992	greenhouse Rome/Italy	symbiont (*D. spinulosum* sp.)
Nostoc sp.	**Mm1**	1992	greenhouse Rome/Italy	symbiont (*Macrozamia* sp.)
A. mendotae x sigmoidea	**04 06**	2004	Březová/Czech republic	fishpond
Anabaena mendotae x sigmoidea	**04 12**	2004	Černiš/Czech republic	fishpond
A. compacta	**04 17**	2004	Dubnenský/Czech republic	fishpond
A. cf. curva	**04 19**	2004	Hejtman/Czech republic	fishpond
A. circinalis x crassa	**04 22**	2004	Husinec/Czech republic	fishpond
A. lemmermannii	**04 24**	2004	Husinec/Czech republic	fishpond
A. circinalis x crassa	**04 26**	2004	Jesenice/Czech republic	fishpond
A. cf circinalis	**04 28**	2004	Hodějovický/Czech republic	fishpond
A. lemmermannii	**04 33**	2004	Orlík/Czech republic	fishpond
A. lemmermannii	**04 38**	2004	Senecký/Czech republic	fishpond
A. cf. flos-aquae	**04 40a**	2004	Skalka/Czech republic	fishpond
A. lemmermannii	**04 42**	2004	Svět/Czech republic	fishpond
Aphanizomenon aphanizomenoides	**04 43**	2004	Svět/Czech republic	fishpond
Anabaena affinis	**04 44**	2004	Svět/Czech republic	fishpond
A. mendotae x sigmoidea	**04 45**	2004	Svět/Czech republic	fishpond
A. cf. spiroides	**04 51**	2004	Svět/Czech republic	fishpond
A. cf. flos-aquae	**04 52a**	2004	Svět/Czech republic	fishpond
A. cf. flos-aquae	**04 53**	2004	Švarcenberk/Czech republic	fishpond
A. circinalis x crassa	**04 56**	2004	Vajgar/Czech republic	fishpond
A. cf. flos-aquae	**04 57**	2004	Vajgar/Czech republic	fishpond
A. circinalis x crassa	**04 59**	2004	Valcha/Czech republic	fishpond
Anabaenopsis cf. elenkinii	**Anaps Plást 05**	2005	Plástovice/Czech republic	fishpond
A. compacta	**Acom Pěšák 06**	2006	Pěšák/Czech republic	fishpond
A. compacta	**Acom Svět 06**	2006	Svět/Czech republic	fishpond
A. lemmermannii - morfotypS	**Alem Lipno 05 silná**	2005	Lipno/Czech republic	fishpond
A. lemmermannii - morfotypT	**Alem Lipno 05 tenká**	2005	Lipno/Czech republic	fishpond
Anabaenopsis cf. elenkinii	**Anaps-Ole 03**	2003	Oleksovice/Czech republic	fishpond
A. eucompacta x reniformis	**Anarenif Pěšák 2**	2006	Pěšák/Czech republic	fishpond
A. eucompacta x reniformis	**Anarenif Pěšák 4**	2006	Pěšák/Czech republic	fishpond
Rivulariaceae	**Litvll06**	2006	Litvínovice - fishpond cascade/Czech republic	fishpond
Anabaena affinis	**Staňk 05-11**	2005	Staňkovský/Czech republic	fishpond

Table 4: List of studied cyanobacterial strains.

2. 3. Culture and brine shrimp (*Artemia salina)* bioassay

Brine shrimp assay was done according to Lincoln 1996. *A. salina* cysts were commercially provided and stored at -20°C before use. 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 concentration of 15-20 individuals ml^{-1}.

100 µl of extracts was transferred into 12-well plate and was kept in laminar box for methanol evaporation. 50 µl of distillated water was added and ultrasonicated for better dissolving of dry extract. 0.95 ml of nauplii (15-20 individuals) was added and number of living and dead individuals and unhatched cysts was counted using stereomicrocope. Individuals were counted again after 24 and 48 hrs and mortality percentage was calculated. Strains causing mortality higher than 50% were considered as toxic.

2. 4. HPLC-ESI-MS analysis

Active extracts with inhibition to *A. salina* ≥ 40% were subjected to high performance liquid chromatography–electrospray ionization-mass spectrometry (HPLC-ESI-MS) analysis. Extracts composition was analyzed on Agilent 1100 series, MSD100 SL-Ion Trap with targeting of ion trap on molecular ions of 900 m/z and on analytical reverse phase column (Zorbax XBD C8, 46 x 150 mm, 5 µm) with flow rate 0.6 ml/min, injection 20 µl and temperature 30°C. The methanol-water separation gradient (Fig. 11) with addition of 0.1 % HCOOH for better ionization in ESI/MS (Electrospray ionization) and total time of 35 min. was used. Molecular ions were determined for each chromatographic peak according to presnce of sodium and potasium adducts and distribution of isotopologues.

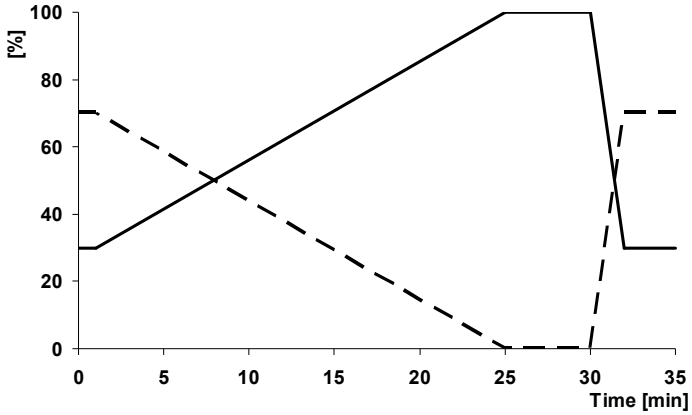


Fig. 11: Standard separation gradient for analytical HPLC. Percent of methanol is marked by solid line, water by dashed line.

2. 6. Preparative HPLC fractionation

Fractionation of selected crude extracts and further brine shrimp bioassay was done in order to find out which compound is responsible for toxic effect. To get the fractions in resonable amounts, preparative HPLC (LabAlliance, Watrex, Prague) was used with the exception of the strain NMB-26, which was separated by analytical HPLC. Fractionation was performed on reverse phase column (C18 Reprosil100, 250x8mm, 5µm, Dr. Maisch GmbH). Standard analytical gradient (Fig. 11) was modified for every selected extract to get better separation conditions. Also different flow rates (2.0 – 3.8ml/min) and different wavelength (λ = 220 – 237 nm) were used (Fig. 12).

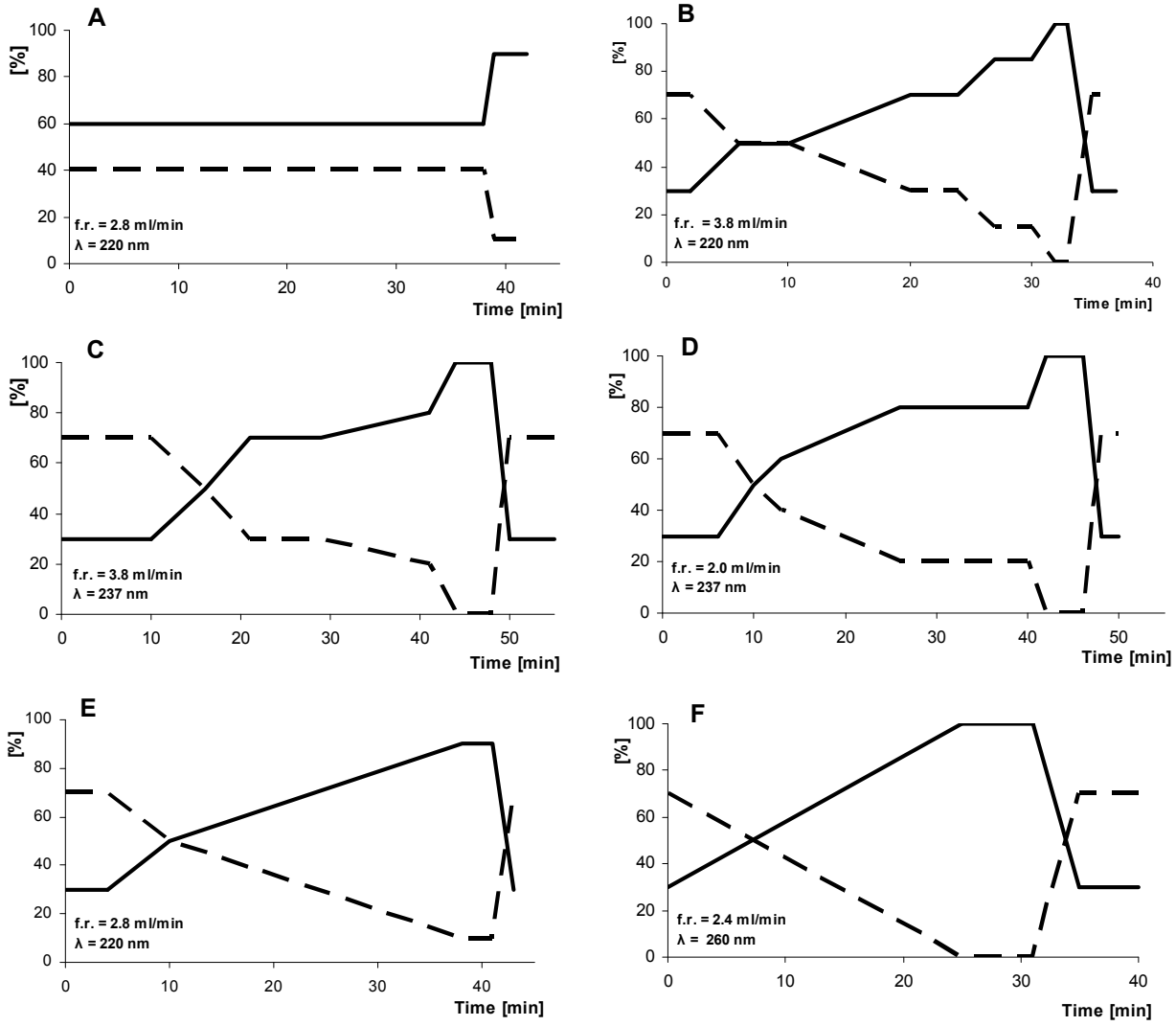


Fig. 12: Separation gradients for fractionation of selected crude extracts on preparative HPLC. **A**: *Nostoc* sp. BR III, **B**: *N. Muscorum* I., **C**: *Cylindrospermum* sp. C24, **D**: *N. ellipsosporum* V., **E**: *Nostoc* sp. Ds1, **F**: *Nostoc* sp. NMB-9. Percent of methanol is marked by solid line, water by dashed line. Flow rates and monitored absorbances are shown in left down corner.

2. 7. Cytotoxicity test on Sp2 cell lines (MTT test)

All strains and fractions were also tested for inhibition on semiadherent murine leukemia cell line Sp/2 by MTT test (Mosman 1983). The cells were kindly provided by Eva Řezníčková and Doc. Jan Kopecký (Institute of Parasitology, CAS, České Budějovice). Tests were performed by Petr Tomek and Kateřina Skácelová as part of their bachelor thesis and also by Pavel Hrouzek. The method is based on ability of living cells mitochondrial enzymes to reduce MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide) to form purple formazan crystrals. Spectrofotometrically measured concentration of formazan is directly proportional to number of proliferating-living cells. The control cells are then used to calculate experimental cells inhibition using equation:

$$\text{Inhibition} = 100 - \left(\frac{(\overline{A_{590} - A_{640}})\ \text{experimental cells}}{(\overline{A_{590} - A_{640}})\ \text{control cells}} \right)$$

2. 8. MALDI-TOF MS analysis

Matrix assisted laser desorption/ionization – time of flight mass spectrometry analysis (MALDI-TOF MS) was performed for several strains as part of broader research in our laboratory. Analyses were performed by Dr. Hans von Döhren (Institute of Biochemistry and Molecular Biology, Technical University Berlin, Germany) on the machine Voyager DE-PRO; PerSeptive Biosystems, Framingham, Mass. Results were used for comparison with our analyses.

3. RESULTS

3. 1. Screening of cyanobacterial toxicity to *A. salina*

Total number of 65 cyanobacterial strains was included in the present study. Investigated cyanobacteria originated from five different habitats (plankton, soil, subaerophytic habitats, periphyton, epiphytic comunities) and strains originated form different symbiotic associations – for exact information about origin and strains isolation see Fig. 11.

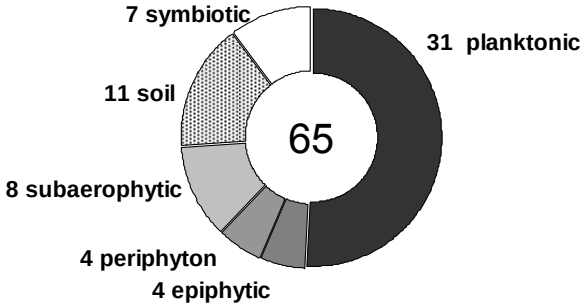


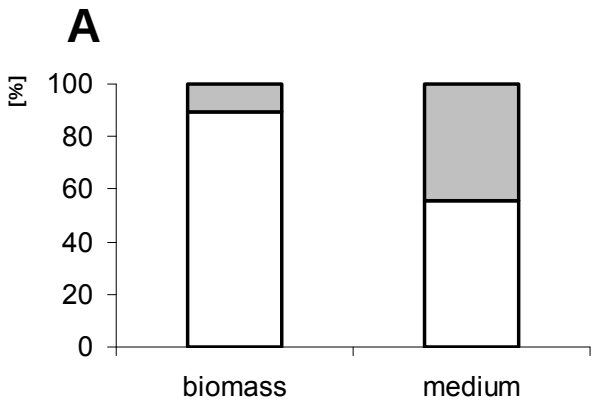
Fig. 11: Origin of tested cyanobacteria.

Toxicity of biomass extracts

Mortality of *A. salina* ≥ 50% was observed in 10.8% of biomass extracts. The highest frequency of toxicity was found in soil strains (27%) whereas only 1 out of 31 (3.2%) planktonic strains contribute to the total number of toxic strains (Fig. 12). Besides high frequency, strong activity was also observed in biomass of soil strains: the most active were *Nostoc* sp. NMB-9 and *N. ellipsosporum* V which caused 100% mortality, *Nostoc muscorum* I (65%) and *Cylindrospermum* sp. C24, *Nostoc* sp. N8 and *Nostoc* sp. NMB-26 caused mortality near 40%. Lower occurances of 14% and 12.5% were found for symbiotic and subaerophytic strains respectively. Nevertheless, symbiotic strain *Nostoc* sp. Ds1 exhibited very strong and fast toxic effect manifested by death of all animals within 24 h. Only one toxic strain was found among epiphytic and periphytic cyanobacteria, however, low number of tested strains from these habitats (four in both) does not allow to formulate relevant conclusions. From these *Cylindrospermum* CyOM isolated from leafs of water plants caused strong toxic effect leading to mortality of 100%.

Toxicity of media extracts

Almost half (44%) of all media extracts exhibited significant toxicity (Fig. 12). Most of them originate from planktonic strains. 74% of all planktonic strains exhibited significant toxic effect. From these, *Anabaena lemmermanii* (100% inhibition), *Anabaena cf. spiroides* 04-51 (91% inhibition) and *Anabaena circinalis/crassa* 04-22 (86% mortality) caused the strongest effect. Identically to biomass extracts, 12.5% of subaerophytic media extracts was considered toxic, however strains with non-toxic biomass extracts were found to produce toxic extracelular compounds. Cultivation media of subaerophytic cyanobacterial samples *Nostoc commune* NC2 and NC3 caused inhibition of 46 and 50%, respectively. No toxic medium extract was found among epiphytic and periphyton strains whereas small number of soil (9%) and symbiotic (14%) strains were toxic to *A. salina* (Fig. 12).



B

Habitat	Biomas toxicity [%]	Medium toxicity [%]
epiphytic	25	0
periphyton	25	0
symbiotic	14	14
soil	27	9
suaerophytic	12.5	12.5
planctonic	3.2	74

Fig. 12 A: Occurrence of toxic strains in biomass and medium extracts of studied strains. Toxic strains are marked by grey colour. **B**: Frequency of toxicity in cyanobacteria from different habitats.

3. 2. Comparison of *A. salina* mortality with Sp/2 cell line inhibition values

Data of *A. salina* mortality were compared with inhibition values of Sp/2 cell line in order to find out if there is some specificity in toxicity of cyanobacteria to *A. salina* or to grazer generally. About 29% of all tested strains were highly toxic to Sp/2 cell lines (cytotoxic) and non toxic to *A. salina* (Fig. 13 – area A). When we focus on area **D** in the Figure 13 we can see that only two strains were toxic to *A. salina* with no activity to cell line. *Nostoc* sp. BR III exhibited strong toxicity to *Artemia* while having marginal effect to cell line. The activity of the strain *Nostoc* sp. Mm1 to *Artemia* is clear, however slightly under the artifitial treshhold value 50%. In this strain no effect to Sp/2 cell line was recorded. In other strains belonging to area **D** (NC7, *N. ellipsosporum* V) strong activity to *Artemia* was found, however it is accompanied by

moderate cytotoxic effect to Sp/2. Strains grouped in the area **B** can not be considered toxic due to their low toxic effect to the both *A. salina* and Sp/2 cell line.

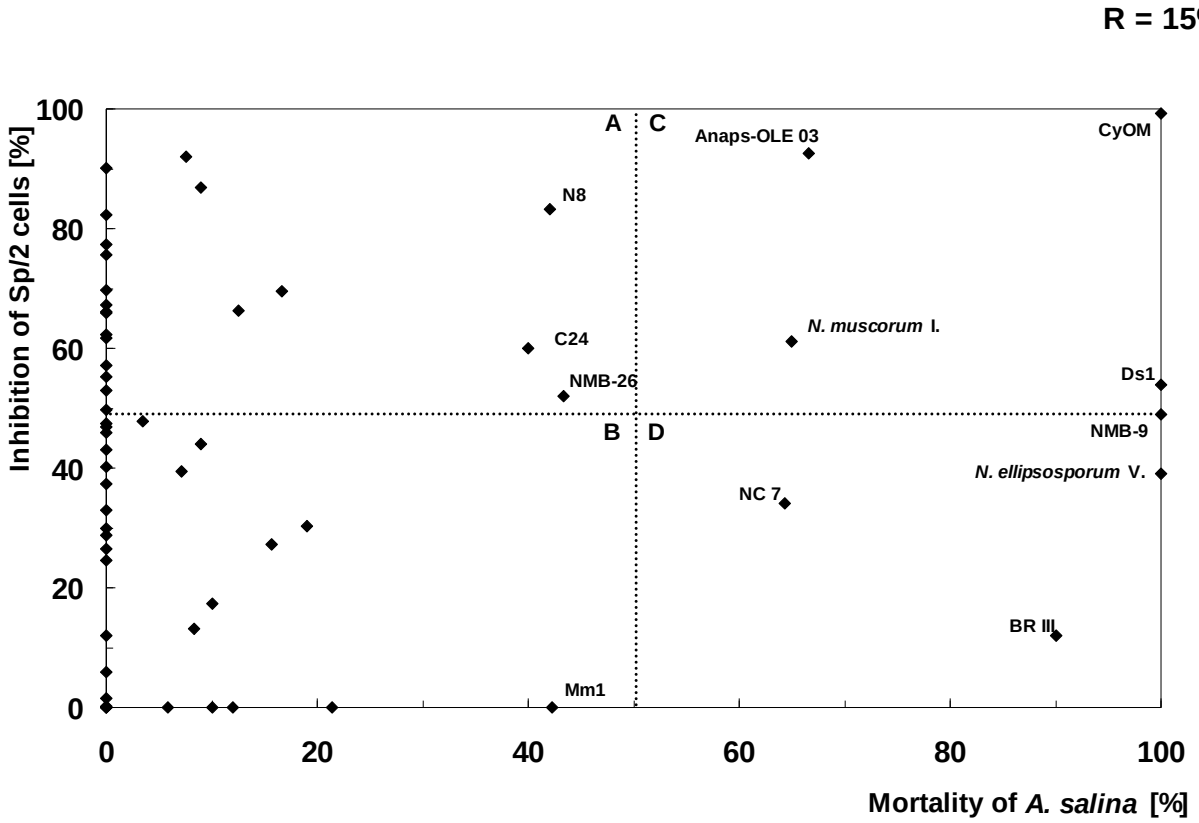


Fig. 13: Graph of correlation of *A. salina* mortality with Sp/2 cells inhibition values. Dotted lines represent borderlines for both *A. salina* mortality and Sp/2 cell lines inhibition values ≥ 50%. Extracts with inhibition of ≥ 50% were considered toxic.

Five of all tested strains (*Cylindrospermum* sp. CyOM, *Anabaenospsis cf. elenkinii* Anaps Ole-03, *N. muscorum* I, *Nostoc* sp. Ds1 and *Nostoc* sp. NMB-9) were found to cause strong damage to both *Artemia* and cell lines (area **C**), however in *N. ellipsosporum* V, N8, NC7, C24, NMB-26, similar effects with inhibition values near the threshold value was found (Table 5). To detect the active compounds we have selected six of these strains (see Table 4) for further fractionation and testing (chapter 3. 4.).

Scientific name	Strain	*A. salina* mortality %	Sp/2 cell line Inhibition (%)
Nostoc sp.	**NMB-9**	**100**	**49**
Nostoc muscorum	**N. musc. I.**	**65**	**61**
Cylindrospermum sp.	**C 24**	**40**	**60**
Nostoc ellipsosporum	**N. ell. V.**	**100**	**39**
Nostoc sp.	**NMB 26**	**43,3**	**52**
Nostoc sp.	**Ds1**	**100**	**54**

Table 5: Strains selected for fractionation and further testing.

3. 3. HPLC-ESI-MS and MALDI-TOF-MS analyses

Table 6 shows list of strains that exerted toxic effect in biomass or medium extract. HPLC-MS analyses were performed for all these strains. MALDI-TOF-MS analyses were performed for strains marked with bold letters and also for strains OSNI 31S01, OBU 36S01, Cam2S01, NMB-27, ISB-13, Cc2, OGU 36S01, De1, Gm1 and Cr4 that did not exert any toxic effect to *A. salina*.

	strains
biomass extracts	BR III, CyOM, **NMB-9**, **NMB-26**, **C 24**, **N. musc. I.**, **N. ellips. V.**, Ds1, **Mm1**, **N8**, NC 7, NC 10, Anaps Ole-03
medium extracts	N. ellips. V., NC 2, NC 3, NMB-26, Gm1, 04-22, 04-26, 04-40a, 04-42, 04-43, 04-44, 04-45, 04-51, 04-52a, 04-53, 04-56, 04-57, 04-59, Anaps Plást05, Acom Pěšák06, Acom Svět06, Alem Lipno05 silná, Alem Lipno05 tenká, Anaps Ole-03, Arenif Pěšák2, Arenif Pěšák4, LitvII, Staňk05-11

Table 6: List of toxic strains.

HPLC-MS chromatograms of biomass extracts for each toxic strain are shown bellow (Fig. 14 - 25). Chromatograms of media extracts are not shown because of low total ion current intensities. Usually, intracellular compounds were present in the media extract but in much lower concentrations. In the HPLC-MS analysis, only spectra between 0-25 min have been analyzed since compounds detected between 25. to 35. min are contaminants (e.g. phthalates), pigments and pigment degradation products (Kopecký, personal communication).

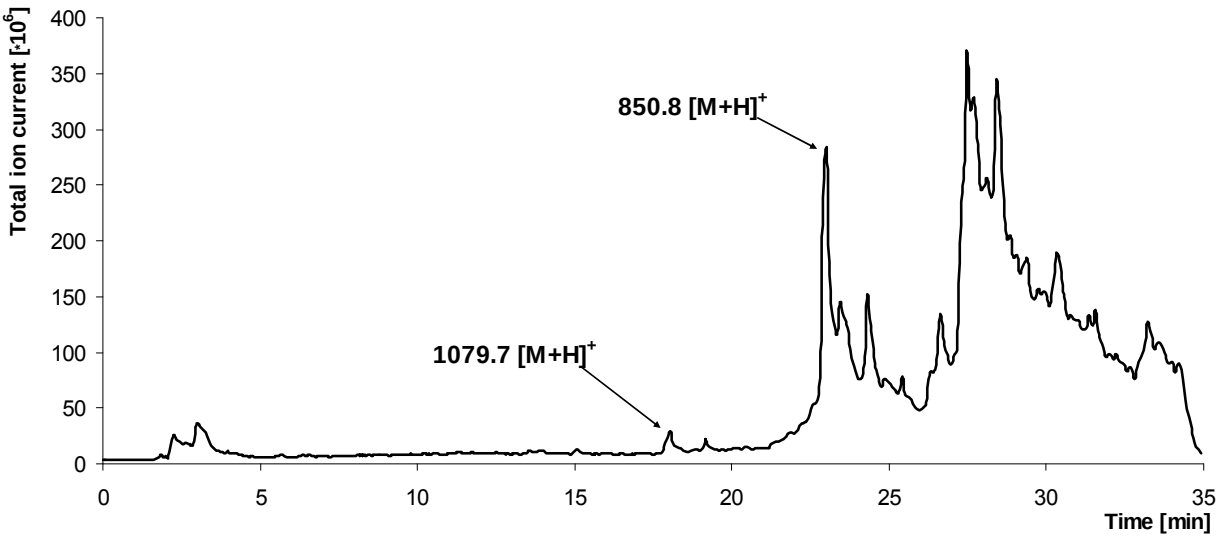


Fig. 14: HPLC-MS chromatogram of the strain *Nostoc* sp. **NMB-9**.

Two molecular ions with MW = 1078.7 and 849.8 were found in the strain *Nostoc* sp. NMB-9 (Fig. 14). The molecular weight of 1078 corresponds to micropeptin 88-B (Ishida et al. 1998), however it exhibited different UV-spectrum (data not shown). The structure of molecular weight 849.8 was confirmed by NMR to be variant of known cytotoxin scytophycin (Tomšíčková, personal communication).

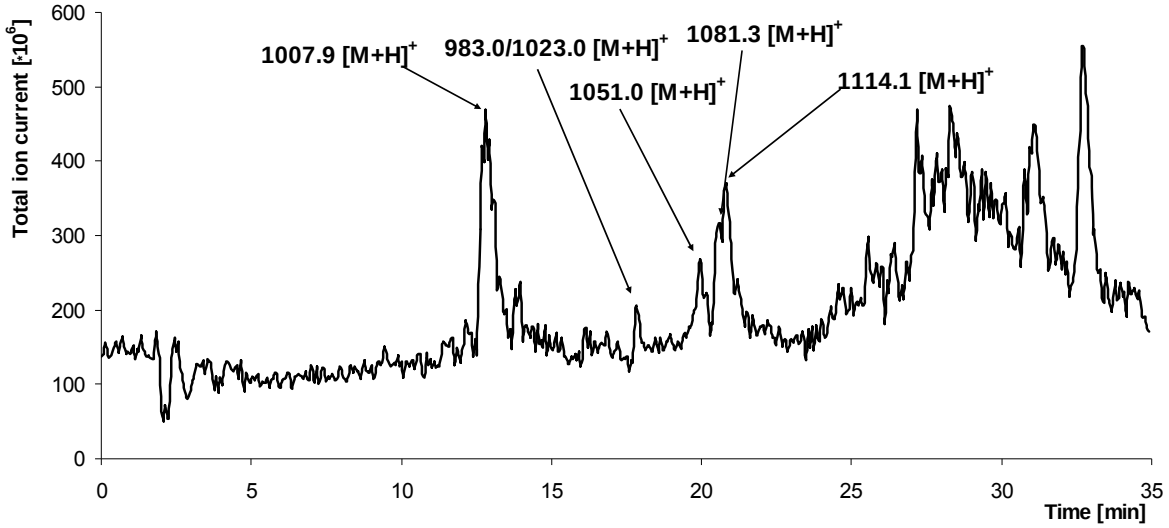


Fig. 15: HPLC-MS chromatogram of the strain *Nostoc* sp. **NMB-26**.

Five clearly recognized compounds were found in the strain *Nostoc* sp. NMB-26. Their molecular weights were 1006.9, 982.0, 1022.0 (known protease inhibitor aeruginopeptin 917-SC, Harada 2001), 1050.0, 1080.3 (cytotoxic structure Nostopeptolide A1, Golakoti 2000) and 1113.1. MALDI-TOF analysis confirmed also presence of microcystin-LA (MW = 910.61), microcystin-RR (MW = 1038.52), microcystin-YR (MW = 1045.47) and aeruginosin (MW = 643.26 $[M+H]^+$).

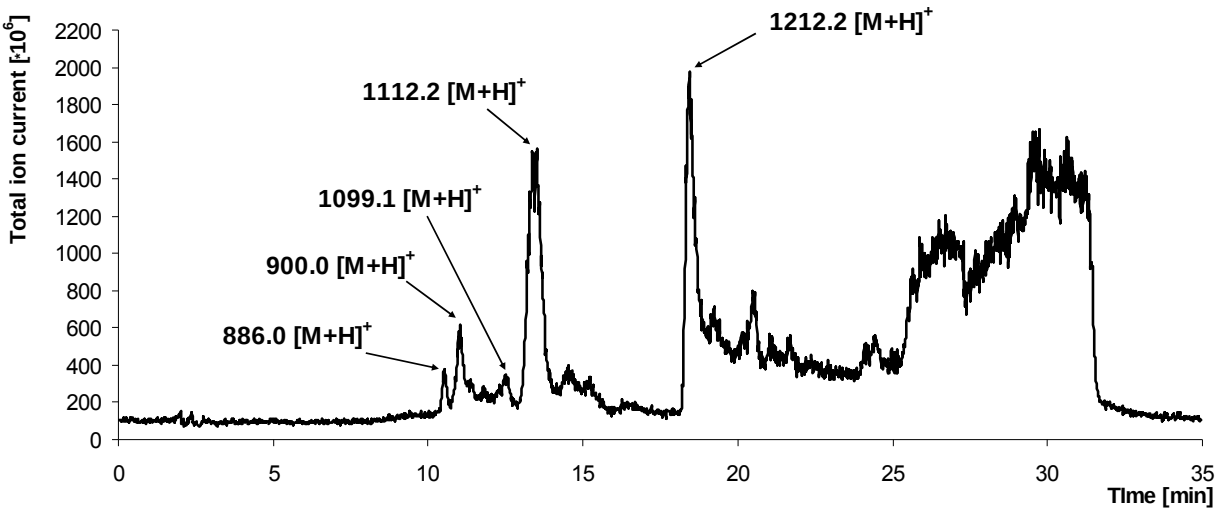


Fig. 16: HPLC-MS chromatogram of the strain *Nostoc muscorum* **I**.

Five compounds with MW = 885.0, 899.0, 1098.1, 1111.2 (Oscillapeptin G, Itou 1999b), 1211.2 were found in the strain *N. muscorum* I. From these only structure of MW = 1211.2 was detected also by MALDI-TOF. These molecular weights are similar to molecular weights of anabaenopeptins and puwainaphycins (Itou et al. 1999a, Moore et al. 1989).

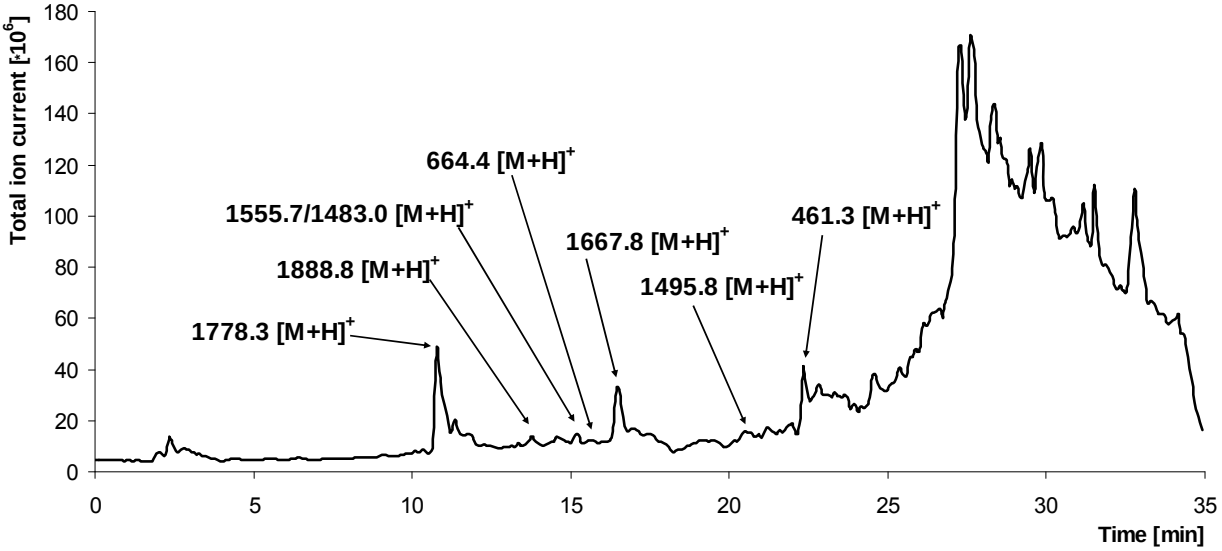


Fig. 17: HPLC-MS chromatogram of the strain *Nostoc ellipsosporum* **V**.

Eight structures with MW = 1777.3, 1887.8, 1554.7, 1482.0, 663.4, 1666.8, 1494.8 and 460.3 were found in the strain *N. ellipsosporum* V. Three of them (1777.3, 1887.8 and 1666.8) were detected also by MALDI-TOF. There is a high number of structures with MW >1000 which is confirmed also by MALDI-TOF. It is very propable that they are unknown types of tetradecapeptides microviridins, which molecular weights ranges between 1500-2000 (Shin et al. 1996).

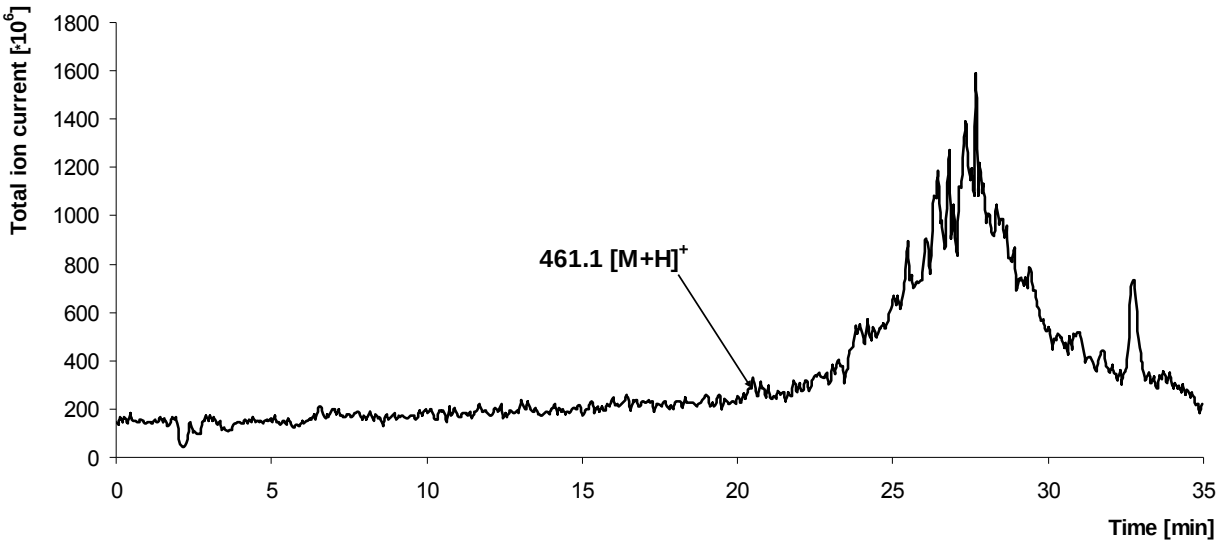


Fig. 18: HPLC-MS chromatogram of the strain *Nostoc* sp.**Ds1**.

We were able to detect only one molecular ion of MW=460.1 in the *Nostoc* sp. Ds1. Nevertheless, it exerted very strong toxicity when tested on *A. salina*. MALDI-TOF analysis is not available for this strain.

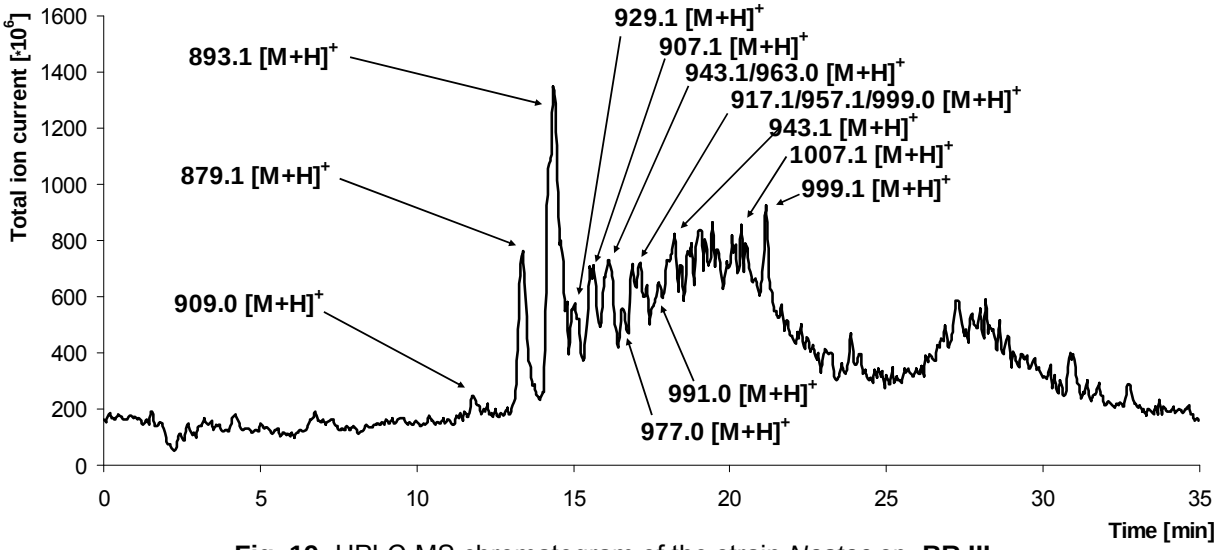


Fig. 19: HPLC-MS chromatogram of the strain *Nostoc* sp. **BR III**.

The extract of strain *Nostoc* sp. BR III is very rich in compounds similar in molecular weight and fragmentation patterns in MS^2 and MS^3. Thus we suggest that they are variant of one class of peptides. 15 compounds with the MW = 908.0, 878.1, 892.1, 928.1, 906.1, 942.1, 962.0, 998.0 ([L-Ser7] mcyst-LR, Fujii et al. 2002), 942.1, 1006.1, 998.1, 990.0 and 976.0 were found. Except 998.0 none of detected compounds was similar to known structure. MALDI-TOF analysis is not available for the strain.

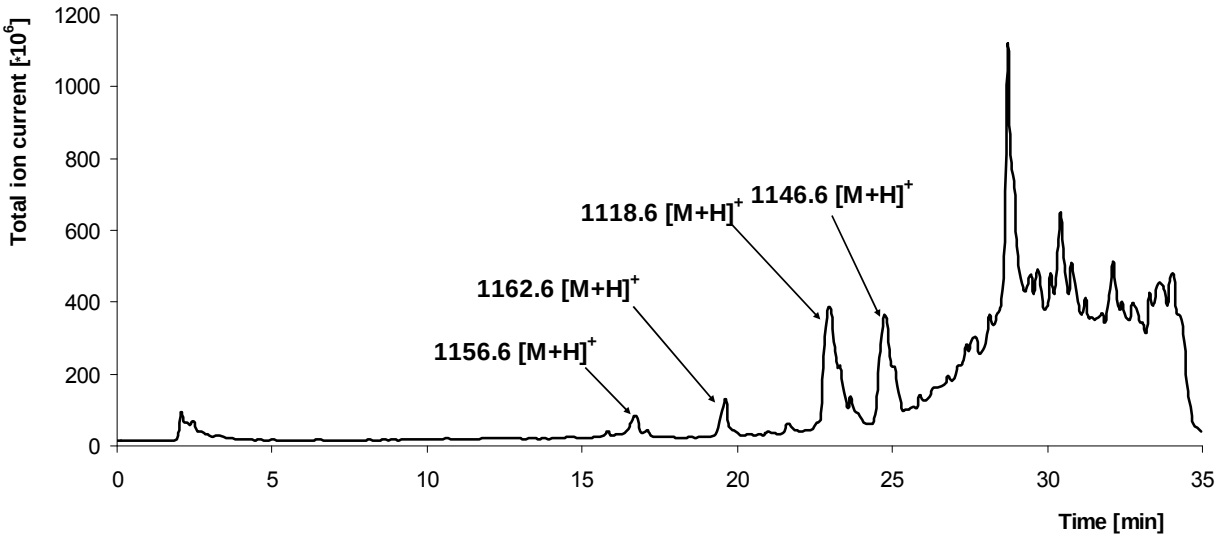


Fig. 20: HPLC-MS chromatogram of the strain *Cylindrospermum* sp. **C 24**.

Strain *Cylindrospermum* sp. C 24 contains four compounds detected by HPLC-MS analysis. Their molecular weights are 1155.6, 1161.6, 1117.6 and 1145.6. None of them is a known structure. Molecule with MW = 1145.6 is novel type of lipopeptide puwaynaphycin (Hrouzek, personal communication). Except structure with MW = 1161.6, all compounds were detected also by MALDI-TOF.

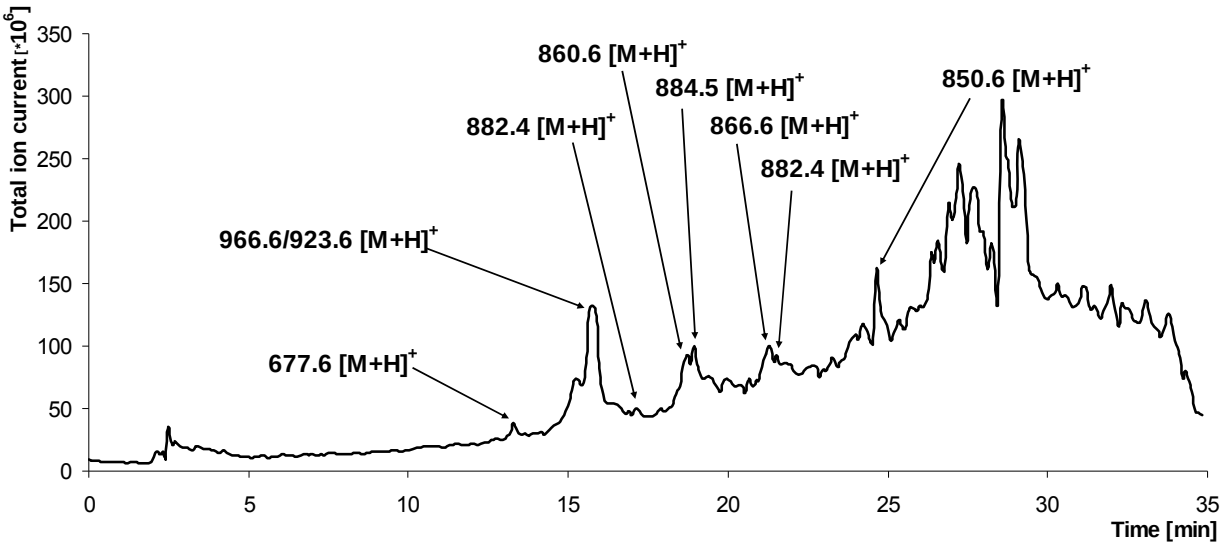


Fig. 21: HPLC-MS chromatogram of the strain *Cylindrospermum* sp. **CyOM**.

Nine clearly recognizable compounds with MW = 676.6, 965.6, 922.6 (Anabaenopeptin H, Itou 1999a), 881.4, 859.6, 883.5 (protease inhibitor micropeptin 88-A, Ishida et al. 1998), 865.6, 881.4 and 849.6 (cytotoxic scytophycin, Tomšíčková, personal communication) were found in highly toxic strain *Cylindrospermum* sp. CyOM. MALDI-TOF analysis is not available for the strain.

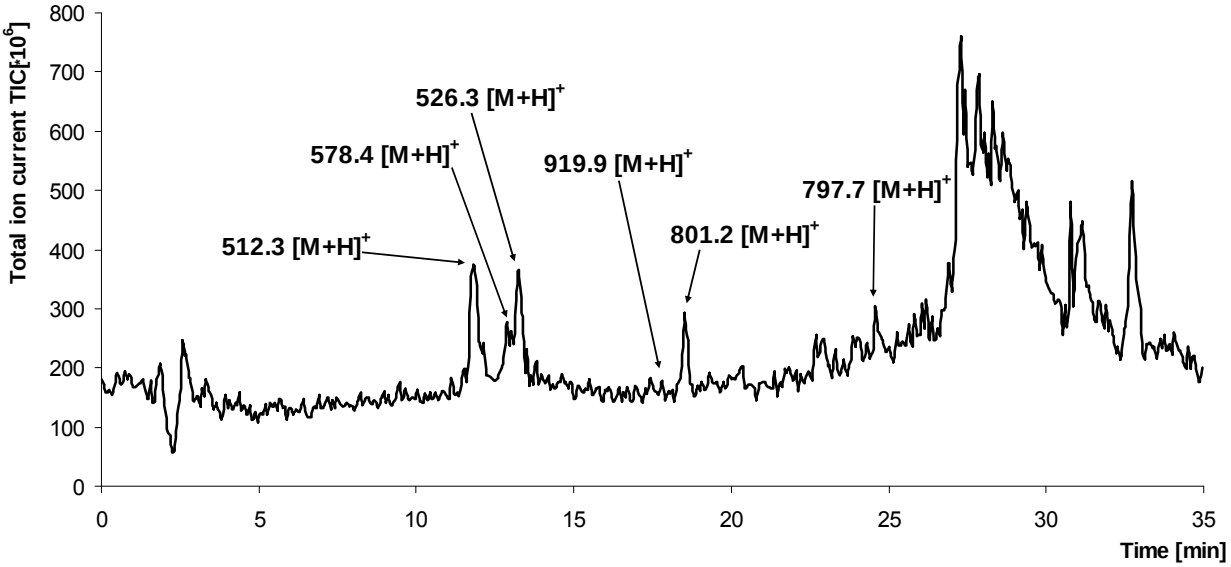


Fig. 22: HPLC-MS chromatogram of the strain *Nostoc* sp. **Mm1.**

Six compounds with MW = 511.3, 577.4, 525.3, 918.9, 801.6 (dimer structure) and 796.7 were found in extract of *Nostoc* sp. Mm1. None of them respond to the known structure. MALDI-TOF analysis revealed also presence of microcystin-YR and -RR with MW = 1145.4 and 1138.45 respectively.

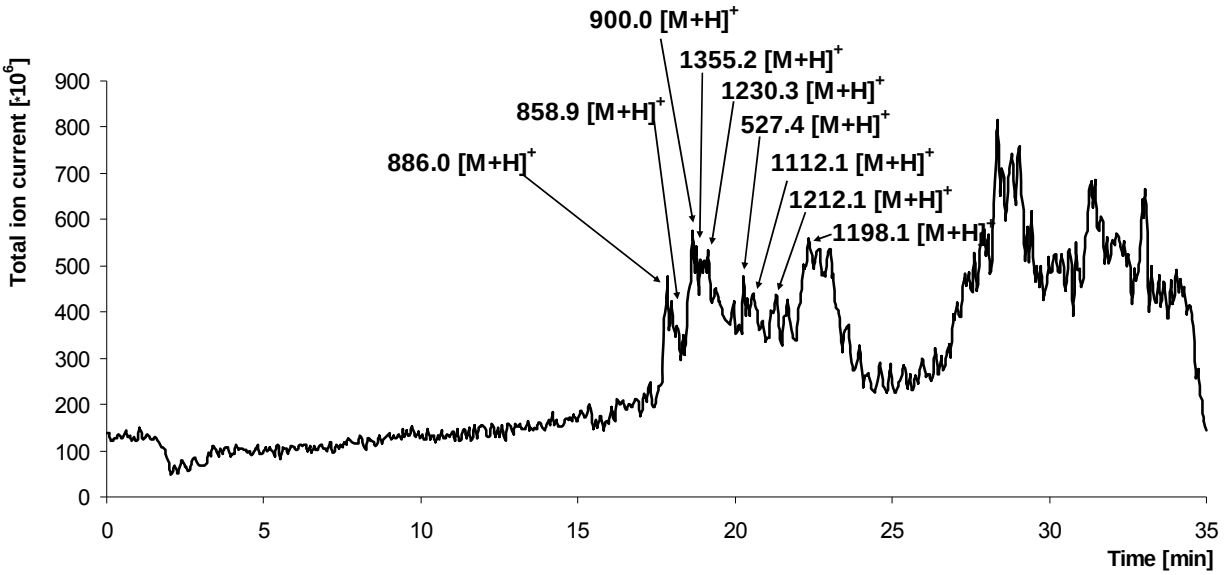


Fig. 23: HPLC-MS chromatogram of the strain *Nostoc* sp. **N8.**

Strain *Nostoc* sp. N8 was found to produce compounds with MW = 885.0, 857.9 (corresponding to oscillamide Y, Sano et al. 1995), 899.0, 1354.2, 1229.3, 526.4, 1111.1 (corresponding oscillapeptin G, Itou et al. 1999b), 1211.1 and 1197.1 respectively. MALDI-TOF analysis confirmed presence of compounds with MW = 899.0 and 1211.1.

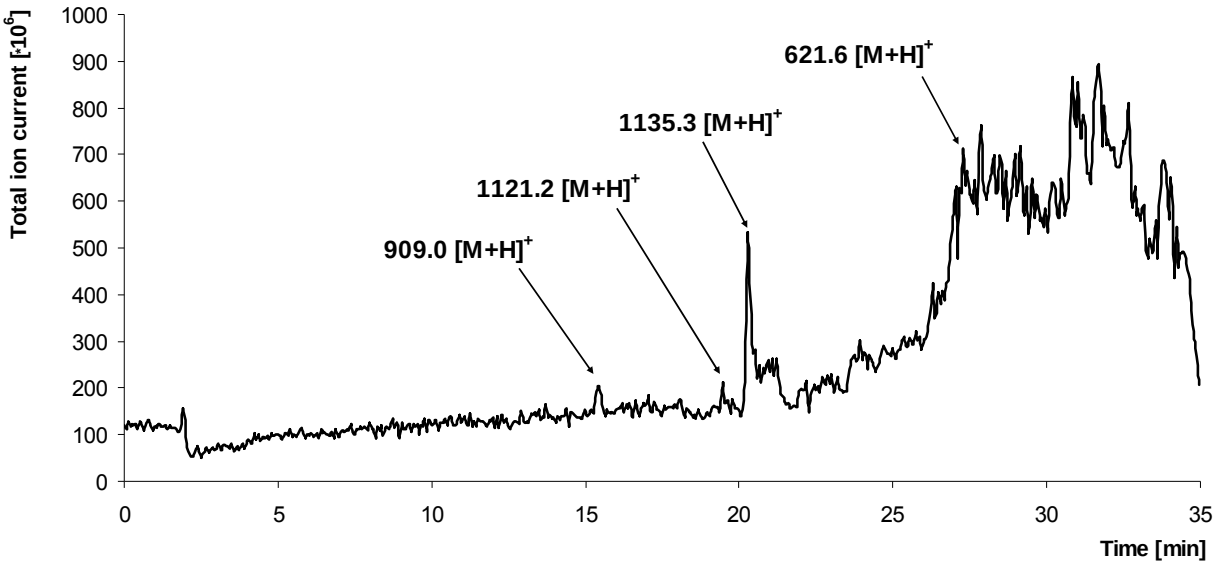


Fig. 24: HPLC-MS chromatogram of the strain *Nostoc* sp **NC7.**

Four distinguishable compounds with MW = 908.0, 1120.2, 1134.3 and 620.6 (strong cytotoxin cryptophycin B, Golakoti 1994) were found in the strain *Nostoc* sp. NC7. MALDI-TOF analyses are not available for the strain.

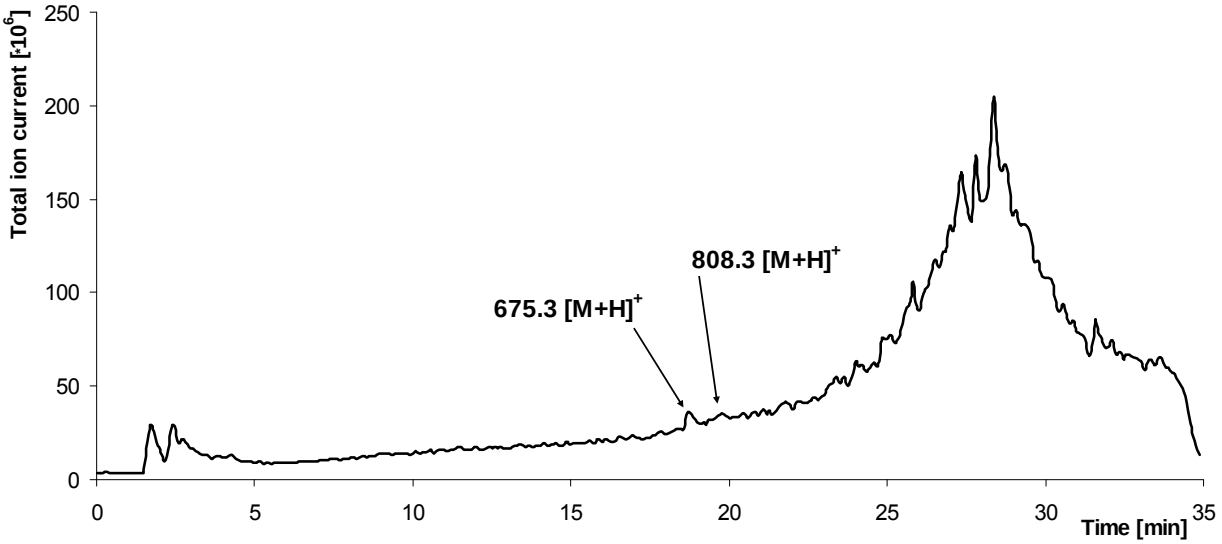


Fig. 25: HPLC-MS chromatogram of the strain *Anabaenopsis cf. elenkinii* **Anaps-Ole 03**.

Only two molecular ions of low intensities were determined in the highly toxic strain *Anabaenopsis cf. elenkinii* Anaps Ole-03 of molecular weights 674.3 and 807.3.

3. 4. Fractionation of selected strains

Strains listed in the Table 5 were fractionated and further tested again on *A. salina* and Sp/2 cell line. On the next figure, there are preparative HPLC chromatograms of the strains *Cylindrospermum* sp. C 24 (A), *Nostoc* sp. NMB-9 (B), *Nostoc ellipsosporum* V. (C) and *Nostoc* sp. Ds1 (D).

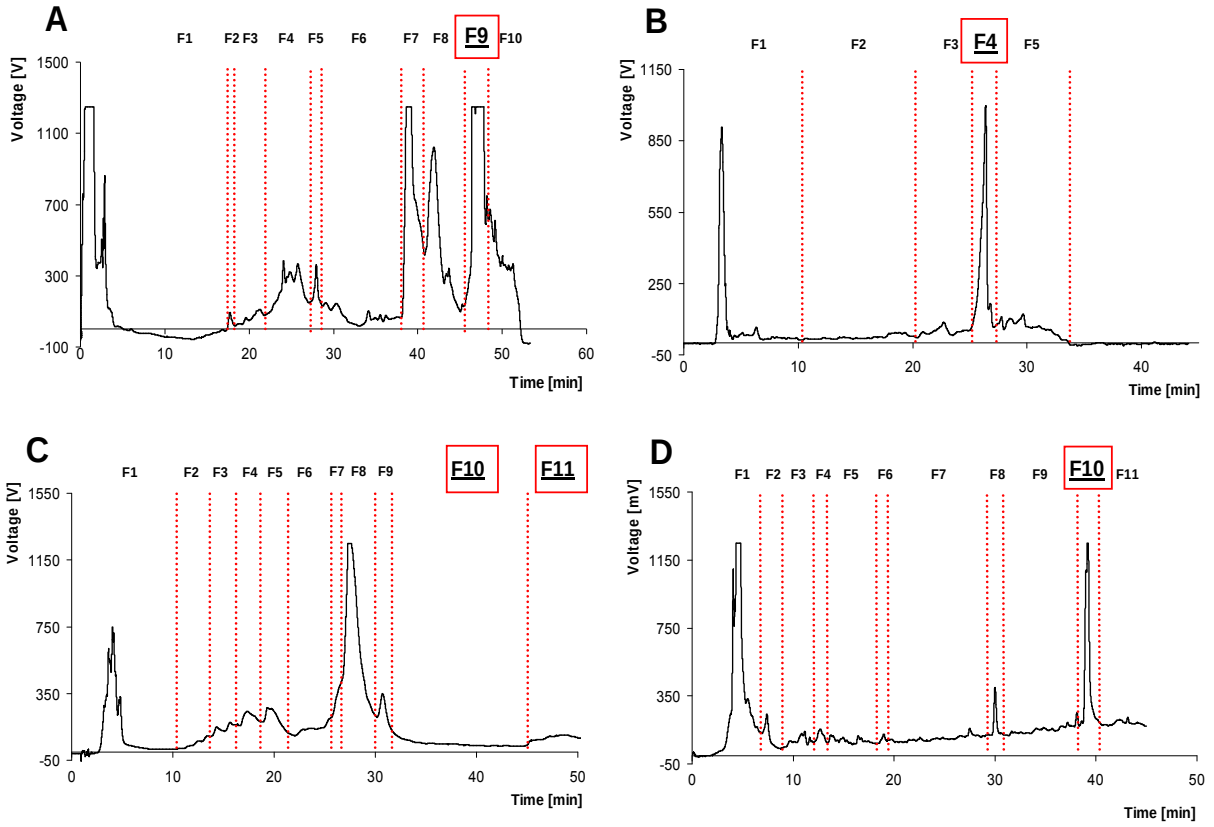


Fig. 26: Preparative HPLC chromatograms. **A**: *Cylindrospermum* sp. C 24, **B**: *Nostoc* sp. NMB-9, **C**: *Nostoc ellipsosporum* V., **D**: *Nostoc* sp. Ds1. Dashed lines represent separation of fractions. Active fractions are underlined and bordered by red square.

Toxicity to *A. salina* and Sp/2 cell line of the strains C 24, NMB-9, Ds1 and *N. ellipsosporum* V. was caused by the same compound for each strain. Fraction number **9** with MW = 1145.6 caused 100% mortality of *A. salina* and 55% inhibition of SP/2 cell line in the strain *Cylindrospermum* sp. C 24. This compound was recently identified as novel type of lipopeptide puwaynaphycin (Hrouzek, personal communication, unpublished). In the strain NMB-9, it was fraction number **4** with MW = 849.6. This supposed to be new type of macrolide lactone scytophycin that exerted very strong (100%) inhibition to the both *A salina* and Sp/2 cell lines. Additionally, also fraction 3 was highly toxic to *A. salina* with mortality 100% (MW = 1078.7).

Fractions **9** and **10** with no UV absorption are responsible for 100% mortality of *A. salina* in the strain *N. ellipsosporum* V. but were only marginally toxic to Sp/2 cell line (19 and 25% inhibition respectively). Compound of MW = 460.1 is responsible for toxic effect of fraction **10** in the strain Ds1. It caused 96% mortality of *A. salina* but was not toxic to cell lines. Fig. 26 shows example of damage of artemid body by fraction 10 (F10) of the strain Ds1. The gut is distorted and feeding apparatus disintegrated. In contrast, the gut of control *Artemia* animal is smooth and body is compact. Distortion of gut was common feature in *Artemia* animals poisoned by toxic cyanobacterial extract.

Fig. 26: Example of damage of artemid body by fraction 10 of the strain Ds1. **A**: treated animal, **B**: control animal (mag.: 200x.).

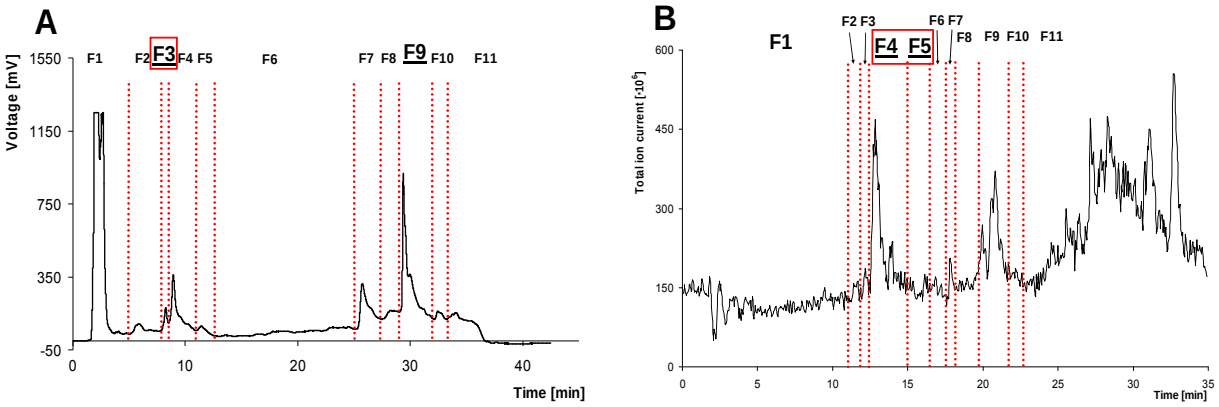


Fig. 27: A: Preparative HPLC chromatogram of the strain *N. muscorum* I., **B**: analytical HPLC chromatogram of the strain *Nostoc* sp. NMB-26. Dashed lines represent separation of fractions. Active fractions are underlined. Fraction toxic to *A. salina* is bordered by red square.

Different compounds were responsible for toxicity in the strains *Nostoc muscorum* I. and *Nostoc* sp. NMB-26 when tested on *A. salina* and Sp/2 cell line. Fraction **3** containing compound of MW = 885.0 caused 61.1% mortality of *A. salina*

in the strain *N. muscorum* I. whereas 79% inhibition of Sp/2 cell line was caused by fraction **9** containing novel cyclic peptide with MW = 1211.6 (Tomek, personal communication).

In the strain *Nostoc* sp. NMB-26, fraction **4** containing compound with MW = 1006.9 caused 40% mortality of *A. salina* and fraction **5**, probably aeruginopeptin 917-B with MW = 1076.1 (Harada 2001), caused 50% mortality of *A. salina*. Different fraction (**1)** caused 30% inhibition of Sp/2 cell line. Probably because of low ionisation, we were not able to determine the molecular ion of the active compound.

4. DISCUSSION

Our data shows that 10.8% of biomass extract of all studied strains exerted toxic effect to *A. salina*. This is in full accordance with Jaki et al. (1999) and Mian et al. (2003), who found toxicity to *A. salina* in 13.9 and 9.1% cases respectively. Bit higher number of toxic strains (25%) was reported by Piccardi (2000) who studied bioactivities of *Nostoc* strains. On the other hand, Falch et al. (1995) reported that 75% of all strains were toxic when tested on *A. salina*, however, only 20 strains were included into this study. Similar results were obtained from study of Rohrlack et al. (2005) who observed toxicity in 70% of strains when tested *Planktothrix* strains on *Daphnia magna*. However, Rohrlack et al. (2000) studied inhibition of daphnid trypsin and not mortality of testing animals. Moreover, studied strains were isolated from the same locality (Lake Zurich) where the probability of isolation clonal strains is high. Such isolates will overestimate the final frequency. Based on these facts we suggest that the overall frequency of strain toxic to *A. salina* ranges between 10-30%.

In further discusion we deicided to join the results obtained in soil and subaerophytic strains, since these habitats exhibited a lot of similar features in ecological conditions and grazing preasure. We found toxicity in 21% out of 19 soil and subaerophytic cyanobacterial strains together. This corresponds to the results of Jaki et al. (1999) who found toxicity in 16.6% of 30 studied 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 also 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 really low number (8, 12 and 8 resp.) of soil and subaerophytic strains and thus the reliable result can not be concluded.

Only 1 out of 31 (3.2%) planktonic strains exerted toxic effect to *A. salina*. Mian et al. (2003) and Piccardi et al. (2000) published very similar results (0% of toxic strains in both) though they worked with low number of strains. In contrast, Falch et al. (1995) found toxicity in 67% of strains but this result is also unreliable due to low number of studied strains. Also Rohrlack et al. (2000) reported toxicity in 70% of planktonic strains. However, he did not investigate mortality of testing animals but inhibition of daphnid trypsin, which is, in fact, more sensitive method since

interactions between inhibitor and purified trypsin are achievable much easier than in whole organism.

Occurrence of toxic strains among symbiotic cyanobacteria was over 14% in the present study, which is in accordance with Piccardi et al. (2000) who observed toxicity in 26% of studied strains.

Overall occurrence of toxicity in media extract was lower then in biomass among soil, subaerophytic, epiphytic and periphytic cyanobacteria. No toxic medium extract was found in epiphytic and periphyton strains whereas only marginal occurrence of toxicity of 14,9 and 12.5% was observed in symbiotic, soil and subaerophytic strains respectively. In contrast, high occurrence of toxic strains was found in planktonic media extracts whereas only one strain was found to be toxic in biomass extract. Similar results were published by Jüttner et Wessel (2003) who found that all five studied strains of *Cylindrospermum* synthesized and excluded zooplankton glucosidases inhibitor DMDP – di (hydroxymethyl)dihydroxypirolidine. Interestingly, the major part of DMPD (80%) was found to be extracellular. We were not able to recognize any compound responsible for toxic effect in the media extracts of planktonic strains because total ion current intensity was very low in chromatograms. We supposed that e. g. neurotoxins as anatoxin-a or –a(s) that are commonly present in planktonic species of *Anabaena* (Carmichael 1979, Mahmood 1986) could cause toxicity, however we were not able to recognize them even with targeting ion trap near the molecular weight around 150. Therefore we suppose the toxicity can be caused by unknown compounds.

Artemia salina toxicity assay has been suggested as a valid method to evaluate the cytotoxic activity (Solis et al. 1993) and thus the method is commonly used as a substitutable assay for screening of cytotoxic compounds. However, Jaki et al. (1999), Mian et al. (2003) and also Berry et al. (2004) found no correlation between *A. salina* mortality and cell lines inhibition values. Our data are in full accordance with these studies. Only 8.5% of all tested strains were toxic to both *A. salina* and Sp/2 cell line. We found out that cytotoxicity is more frequently observed feature then toxicity to invertebrate among cyanobacteria. Therefore, we suggest that it is not possible to investigate cytotoxicity using *A. salina* bioassay.

Scientific interest in cyanobacteria – grazer interactions raises the question whether cyanobacteria from specific habitats possess the ability to produce secondary metabolites as a result of grazer pressure on it. For example, whether

planktonic cyanobacteria living in water produce and exclude toxins to “avoid to be eaten” by grazers. On the other hand, production of extracelular toxin to soil environment lack sense because of difficult diffusion through ecosystem. Consequently high frequency of cyanobacteria with intracellular toxin should be preferred in soil environment which is in accordance with our data. Our data revealed that planktonic cyanobacteria produce and are able to exclude toxins. Over 74% of media extracts of planktonic strains were found to be toxic to *A. salina* whereas only one strain was found toxic in biomass extract. In contrast, only 6% of media extracts of soil and subaerophytic strains together were toxic. It is logical to suppose that planktonic cyanobacteria exclude toxins as a defense against grazers whereas soil and subaerophytic cyanobacteria do not exclude toxins but are keeping them inside the cells.

On the other hand, our correlation data revealed that toxicity of cyanobacteria to *A. salina* is not specific. Only strains *Nostoc* sp. Mm1 and *Nostoc* sp. BRIII were found to exhibit specific toxicity to *A. salina* whereas had no effect to Sp/2 cell line. This fact is supported also by fractionation of strains highly toxic to both *A. salina* and Sp/2 cell line. In four of six fractionated strains, the same compound was responsible for toxic effect to both *A. salina* and Sp/2 cell line respectively. Only in the strains *N. muscorum* I. and *Nostoc* sp. NMB-26, different compound was found to cause toxic effect to *A. salina* and Sp/2 cell line. Moreover, in the strain *Nostoc* sp. BR III, no particular toxic compound was found although the strain was highly toxic. It can be due to synergic effect of many compound present in the strain. Therefore we suggest that these compounds are not synthesized specifically against grazers but are highly toxic in general. However, they can play a role in defensive mechanism of cyanobacteria against grazer and can be more frequent in habitats with higher predation preasure.

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