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Production of cyanobacterial toxins from two *Nostoc* species (*Nostocales*) and evaluation of their cytotoxicity *in vitro*

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ABSTRACT

Cyanobacteria are among the oldest autotrophic organisms with cosmopolitan distribution and known as producers of secondary metabolites with toxic properties named "cyanotoxins". Studies with respect to toxin production of genus *Nostoc* are yet limited. In the present study we have investigated two *Nostoc* species (*Nostoc linckia* and *Nostoc punctiforme*) for production of intracellular and/or extracellular compounds with cytotoxic potential. Extracts and algal growth media were assessed by different *in vitro* tests using freshly established mouse primary cultures from different tissues and one fish cell line. Our data showed that the mouse cells are more sensitive to toxic compounds than the fish cells. Both *Nostoc* species produced intracellular and extracellular bioactive compounds with different effects on mouse and fish cells. The presence of cyanotoxins as anatoxin-*a* and microcystins/nodularin was confirmed by HPLC and ELISA analyses. Therefore, *Nostoc* species are not only sources of bioactive compounds with therapeutic action, but they can be a potential hazard to aquatic systems as well as to animal and human health.

Key words: *Cyanobacteria*, *Nostoc*, cytotoxicity, *in vitro*, toxins

Introduction

Blue-green algae (*Cyanobacteria*) are among the oldest autotrophic life form of the earth. They have cosmopolitan distribution and can be found in different habitats, including Antarctic lakes and thermal springs. Most commonly they are known as planktonic members of the marine and freshwater environments. *Cyanobacteria* are increasingly gaining importance in view of health hazards and ecological risks caused by the secondary metabolites with toxic properties named "cyanotoxins". Cyanotoxins cause direct intoxications of animals and humans through contact with bloom water or indirect poisoning due to consumption of contaminated food (Jochimsen et al., 1998; Falconer, 1999; Ito et al., 2000). The most common and well-studied producers of cyanotoxins are *Microcystis aeruginosa*, *Aphanisomenon flos-aquae*, *Anabaena flos-aquae*, *Cylindrospermopsis raciborskii*, *Planktothrix agardhii*, *Lyngbya majuscula*, *Nodularia* and *Oscillatoria* (Hitzfeld et al., 2000; Lakshmana Rao et al., 2002).

On the other hand, *Cyanobacteria* are known to produce bioactive compounds with a wide variety of biological activities as immunosuppression, inhibition of different enzymes, antiviral, antifungal and anticancer activity (Namikoshi & Rinehart, 1996).

There is limited number of studies conducted on the toxic potential of genus *Nostoc*, which has cosmopolitan distribution and can be occurs in both terrestrial and aquatic ecosystems.

So far, only three *Nostoc* strains are known to produce microcystins. Sivonen et al. (1990) reported *Nostoc sp.* strain 152 as a producer of nine hepatotoxic peptides with chemical and toxicological properties similar to those of the hepatotoxic hepta- and pentapeptides produced by other *Cyanobacteria*. Five of these peptides were identified as new types of microcystin-LR homologs (Sivonen et al., 1990).

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Nostoc sp. strain DUN901 isolated from brackish water of Barrow Ski Club Lake in the United Kingdom is another producer of microcystins (Beattie et al., 1998). A lichen associated terrestrial *Nostoc sp.* strain IO-102-I was also reported to produces six rare forms of microcystins (Oksanen et al., 2004).

As root symbionts of cycad trees (*Cycas micronesica*), specimens of the genus *Nostoc* were found to produce β-methylamino-L-alanine (Cox et al., 2003; Murch et al., 2004), a neurotoxic nonprotein amino acid, which is associated with amyotrophic lateral sclerosis/Parkinsonism dementia complex. Cytotoxic antiviral indolocarbazoles (6-cyano-5-methoxy-12-methylindolo[2,3-alpha]carbazole and 6-cyano-5-methoxyindolo[2,3-alpha]carbazole) were isolated from *Nostoc sphaericum* EX-5-1 (Knubel et al., 1990). According to the authors these compounds have weak cytotoxic effects on KB and LoVo human carcinoma cells and antiviral activity against HSV II. Gustafson et al. (1997) have been isolated a new anti-HIV protein (cyanovirin-N) from *Nostoc ellipsosporum*.

The number of bioactive compounds isolated or produced from *Nostoc* species is increasing continuously. Cryptophycins are anticancer agents isolated from terrestrial *Nostoc* strains (Smith et al., 1994; Chen et al., 1998; Eggen & Georg, 2002). Their antiproliferative and antimitotic activity is due to binding to the tubulin molecules, inhibiting tubulin assembly and disassembly. A cyclic peptide (nostocyclamide M) with allelopathic activity was isolated from *Nostoc* 31 (Juttner et al., 2001). The authors suggested this strain as a source of natural pesticides.

In this study, we have examined whether two *Nostoc* species (*Nostoc linckia* and *Nostoc punctiforme*) are capable to produce intracellular and/or extracellular compounds with cytotoxic potential. Investigations were based on analitical, immunobiological and *in vitro* cell assays.

Materials and Methods

Cyanobacterial cultures and preparation of extracts

Two different freshwater species from genus *Nostoc* (*Nostocales, Cyanobacteria*) were studied: *Nostoc linckia* (Roth.) Born et Flah – kept in PACC (Plovdiv Algal Culture Collection) under No 5085 and *Nostoc punctiforme* (Kütz) Har – kept in PACC under No 8646.

Blue-green algae were grown intensively under sterile conditions using a Z-nutrient medium. Cultures were synchronised by altering light/dark periods of 16/8 hours. The temperature was 33°C and 22°C during the light and dark period, respectively. The intensity of light during the light period was 224 μmol photon $s^{-1}m^{-2}$ (Lux 12000). The culture medium was aerated with 100 litres of air per hour per one litre of medium, adding 1% CO_2 during the light cycle. The period of cultivation was 14 days.

Extracts of the blue-green algae were obtained according to the method of Krishnamurthy et al. (1986) with slight modifications. Briefly, *Nostoc* species were removed from the Z-medium and weighed, then frozen and thawed, and extracted twice (3 h and overnight) with water-methanol-butanol solution (15:4:1, v:v:v, analytical grade) at 22°C while stirring. The extracts were centrifuged at 10000 rpm for 30 min. The supernatants of the two extracts were pooled and organic solvents removed via speed-vac centrifugation (SAVANT, Instruments Inc. Farmingdate, NY, USA) at 37°C for 2 h The resulting extract was sterilized by filtration through a 0.22 μm Millipore filter and prepared to give equivalent final concentrations of 150 mg/ml (wet weight/volume) suspended algal matter.

To investigate whether *Nostoc* species release toxic products into their culture environment, the nutrient solution in which the algae were cultivated during the 14 days was filtered through a 0.22 μm Millipore filter. The final equivalent concentration of suspended algal matter per mL culture medium was 20 mg/ml (wet weight/volume). This algal medium was tested for cytotoxicity *in vitro.*

Animal cell cultures

Four different primary mouse cell cultures were used for the cytotoxicity tests - kidney cells, skin fibroblasts, thymus fibroblasts and endothelial cells as well as one fish cell line named RTgill.

Mouse cells were cultured in 75 cm^2 flasks in Dulbecco's Modified Eagle's Medium (DMEM, Gibco™, Paisley, Scotland, UK), supplemented with 10% (v/v) heat inactivated fetal calf serum (FCS, PAA Laboratories GmbH, Linz, Austria), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma, Steinheim, Germany), at 37°C with 5% CO_2 in air and high humidity. Cell viability was measured with the trypan blue exclusion test (Berg et al., 1972) prior to seeding.

Fish RTgill cells were cultured as originally described by Lee et al. (1993) in an atmosphere of air in 75cm^2 Nunc culture flasks at 19°C in Leibovitz's L-15 medium without phenol red (Invitrogen, Karlsruhe, Germany), supplemented

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with 5% fetal bovine serum, FBS (Biochrom, Berlin, Germany) and penicillin-streptomycin (20 U/mL – 20 μg/mL, Biochrom, Berlin, Germany).

Exposure conditions

Prior to exposure, cells were plated in 96-well tissue culture plates at a density of $1.5x10^4$ per 200 μL DMEM medium with 10% FCS for mammalian cells and $5x10^4$ cells per 200 μL L-15 medium with 5% FBS for the piscine cell line. After 24 h of attachment, the medium was removed and replaced by the exposure medium as described below.

Exposure to the cyanobacterial extracts

Mouse cells were exposed to three concentrations of the algal extracts – 2.5%, 5% and 10%. These concentrations were obtained by adding 5 μL, 10 μL and 20 μL of the extract to 195 μL, 190 μL and 180 μL DMEM culture medium with 10% FCS. Control wells were prepared by adding 10 μL Millipore water to 190 μL culture medium. The cells were exposed to the cell extracts for 24 or 48 h prior to analysis of cytotoxicity by the MTT assay.

The piscine cell line was exposed to varying concentrations of the algal extracts with the highest concentration being equivalent to an extract of 15 mg/mL suspended algal matter. Exposure was done in L-15 medium in the absence of serum. The exposure temperature was 19°C as for routine maintenance. Cytotoxicity of the algal extracts was assessed after 24h of exposure by a combination of the alamarBlue™/CFDA-AM and Neutral Red cytotoxicity assays as described below.

Exposure to the cyanobacterial growth media

In addition to exposure to algal extracts, the cells were also exposed to varying concentrations of media in which *Nostoc* species have been grown for 14 days. Mammalian cells were treated with algal medium at final concentrations of 2.5% (5 μL of the algal medium to 195 μL DMEM), 5% (10 μL of the algal medium to 190 μL DMEM) and 10 % (20 μL of the algal medium to 180 μL DMEM) under the conditions mentioned above. A similar concentration of Z-medium was used as appropriate control. The highest concentration for the fish cell line was 50% of the algal medium, which was obtained by adding 100 μL of the algal Z-medium to 100 μL of L-15 medium in the absence of serum. Cytotoxicity of algal growth media was assessed after 24h of exposure by a combination of the alamarBlue™/CFDA-AM, Neutral Red and MTT cytotoxicity assays as described below.

Cytotoxicity assays

MTT test

The MTT (3-(4',5'-dimethylthiazol-2'-yl)-2,5-diphenyltetrazolium bromide, Sigma, St. Louis, MO, USA) assay was carried out in accordance with Edmondson et al. (1988). After the desired time of contact with algal extracts (24 or 48 h), 20 μl of a 0.5% (w/v) solution of MTT in PBS were added directly to each well and incubated at 37°C for 4h in the dark. After incubation, the medium with the dye was aspirated and plates inverted to drain unreduced MTT, and 0.1 ml of 0.04 mol/L HCL in isopropanol was added to each well in order to facilitate solubilization of the formazan product. The plates were shaken, and absorbance was read at 570 nm.

Alamar Blue™and CFDA-AM

Two fluorescent indicator dyes were used in combination as previously described using L-15/ex as a simplified culture medium (Schirmer et al., 1997). The two dyes were alamar Blue™ (BioSource, Solingen, Germany) which (similar to MTT) is a measure of the redox potential of a cell, and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM, Molecular Probes, Eugene, OR, USA), which measures cell membrane integrity. After exposure of the cells to algal extracts or algal media, wells were emptied and filled with 100 μL of a mixuture of 5% v/v alamar Blue™ and 4 μM CFDA-AM in L-15/ex and incubated in the dark for 30 min prior to fluorescent measurement. Fluorescence was analyzed using a SPECTRAmax Gemini spectrophotometer (Molecular Devices, Munich, Germany) at optimized respective excitation/emission wavelengths for alamar Blue™ and CFDA-AM of 530/595 nm and 493/541 nm, respectively.

Neutral Red test

The neutral red working solution was prepared prior to each cytotoxicity test by diluting the stock solution 1:100 in L-15/ex to yield 50 μg neutral red in 1 ml L-15/ex. This working solution was filter-sterilized with a 0.2 μm Millipore filter to remove fine precipitates of the dye. For immediate measurements, aliquots of 100 μl of this working solution were added to the culture plates. After an incubation period of 1h, which allowed the dye to be taken up by cells with intact lysosomes, the dye solution was removed and the wells

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rinsed with 100 µl of a mild fixative, containing 0.5% v/v formaldehyde and 1% w/v $CaCl_2$ in ddH_2O. This rinsing step removed any excess neutral red that had not been localized in lysosomes during the incubation period. In order to solubilize lysosomal neutral red, an aliquot of 100 µl of on extraction solution (1% v/v acetic acid and 50% v/v ethanol in ddH_2O) was then added to each well. The plates were placed on an orbital shaker at approximately 40 rpm before fluorescence was measured 10 min later. Fluorescence was quantified with the SPECTRAmax Gemini spectrophotometer (Molecular Devices, Munich, Germany) at respective excitation and emission wavelengths of 530 and 645 nm.

High performance liquid chromatography (HPLC) analysis

Chemicals and standards

HPLC Super gradient acetonitrile was purchased from Lab-Scan Analytical Sciences (Dublin, Ireland) and ammonium acetate from Scharlau Chemie S.A. (Barcelona, Spain). Water used for HPLC was purified with a Milli-Q plus PF system (Millipore, Molsheim, France).

Anatoxin-*a* (AnTx-*a*) was purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), microcystin-LR (MC-LR) was from BIOMOL GmbH (Hamburg, Germany) and saxitoxin (STX) was from R-Biopharm GmbH (Darmstadt, Germany).

HPLC conditions

Chromatography was performed with an ÄKTA™ explorer 100 Air system (Amersham Pharmacia Biotech AB, Uppsala, Sweden) using an UNICORN V4.00 software. The analytical column was a Discovery® C_{18} (5x4 mm I.D., 5 µm) from Supelco (Bellefonte, PA, USA). The mobile phase consisted of a mixture of solvent A (10 mM ammonium acetate, pH=5.5) and solvent B (10 mM ammonium acetate-acetonitrile, 80:20, v/v) as follows: 0% of B at 0 min, 100% of B at 45 min to 65 min using a linear gradient. Flow-rate was 0.8 ml/min and UV detection was performed at 238 nm. All runs were carried out at room temperature. The column was reequilibrated with 8 ml of the solvent A between runs. Each standard was run separately (AnTx-*a* 5 µg/ml, MC-LR 5 µg/ml, STX 40.5 pg/ml, 200 µl injection volume) and thereafter a mixture of all standards with the same concentrations in 200 µl was run again. 200 µl of each sample were injected for HPLC analysis. Toxins and their concentrations in the samples were determined by comparing retention times and peak areas for each toxin with those of the standards.

ELISA

Saxitoxins

The samples were analyzed by the Ridascreen™ saxitoxin ELISA kit (R-Biopharm, Darmstadt, Germany). This is a competitive ELISA for the quantitative analysis of saxitoxin and related toxins based on the competition between the free toxins from samples or standards and an enzyme-conjugated saxitoxin for the same antibody. The mean lower detection limit of the Ridascreen™ saxitoxin assay is about 0.010 ppb.

Microcystins

Analysis of samples was performed using the Microcystin Plate kit (EnviroLogix Inc., Portland, USA.). As for the saxitoxin ELISA, this a quantitative, competitive immunosorbent assay. The limit of detection of the EnviroLogix Microcystin Plate kit is 0.05 ppb..

Results and Discussion

In vitro toxicity of Nostoc extracts

Cytotoxic activity of the extracts was assessed by different *in vitro* tests using freshly established mouse primary cultures from different tissues and one fish cell line.

After treatment of the mouse cells with varying concentrations of extracts from *Nostoc linckia* and *Nostoc punctiforme* distinct responses were detected depending from the origin of the cells and time of exposure (Figure 1). The cell viability (as measured by MTT) was weakly affected in almost all cell cultures after 24 h of exposure. A greatest cytotoxic effect (from 40% to 60 %) was observed for both *Nostoc* extracts 48h after treatment with 10% of the extracts (Figure 1). This effect was dose-dependent only for the kidney cells, which indicates that these cells are the most sensitive system from all tested here.

To characterize the influence of *Nostoc* extracts on fishes, we have used a fish cell line RTgill. Fish cells were treated with 0.5%, 2.5% and 10% of the extracts for 24 hours. This treatment has been done in the absence of serum as we have previously established that fish cells are more sensitive to cyanotoxins if serum is absent (Teneva et al., 2003). The combination of three cytotoxicity tests (Alamar Blue, CFDA-AM and Neutral red) allowed indication of different levels and mechanisms of cell stress caused by the investigated *Nostoc* extracts..

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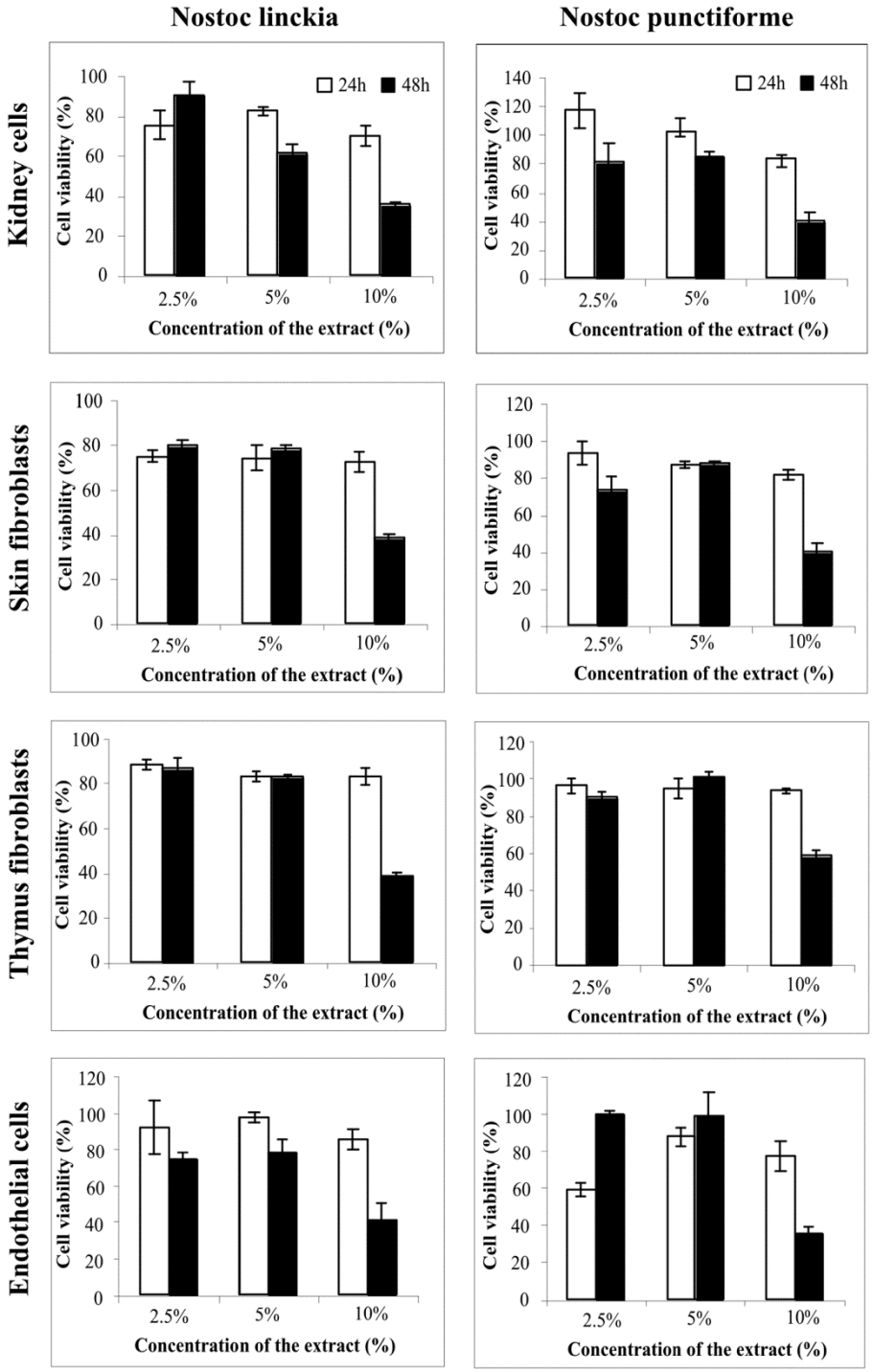

Figure 1. *Viability of mouse cell cultures treated with Nostoc extracts for 24h (white bars) or 48h (black bars) as determined by MTT assay. Cells were exposed to equivalent concentration of suspended algal matter of 3.75 mg/ml (2.5% of extracts), 7.5 mg/ml (5% of extracts) and 15 mg/ml (10% of extracts). After exposure, MTT was applied as described in Materials & Methods and the absorbance of the formazan product assessed at 570 nm. Absorbance readings were expressed as percentage (%) of the readings in the cultures receiving Millipore water as the control. Data are represented as mean values of triplicates ± SD.*

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In contrast to mouse cells, treatment of fish cells with *Nostoc linckia* and *Nostoc punctiforme* extracts led to different effects (Figure 2). Alamar blue and CFDA-AM tests showed no effect or very weak cytotoxicity of *Nostoc linckia* extract while the neutral red assay showed decrease of cell viability to 60% as compared to the control in a dose-dependent manner (Figure 2). These data indicate that *Nostoc linckia* extract influence the lysosomal integrity, whereas mitochondria and cell membrane were not affected. A detected stimulatory effect at lower concentration of the extracts is common and well-known phenomenon for the toxic compounds. Interestingly, treatment of fish cells with low concentration of *Nostoc punctiforme* extract had a cytotoxic effect, while higher concentrations had a stimulatory effect (Figure 2). These effects were observed by all used viability assays. The results show that the two investigated *Nostoc* extracts contain different bioactive compounds with different effects on mouse and fish cells.

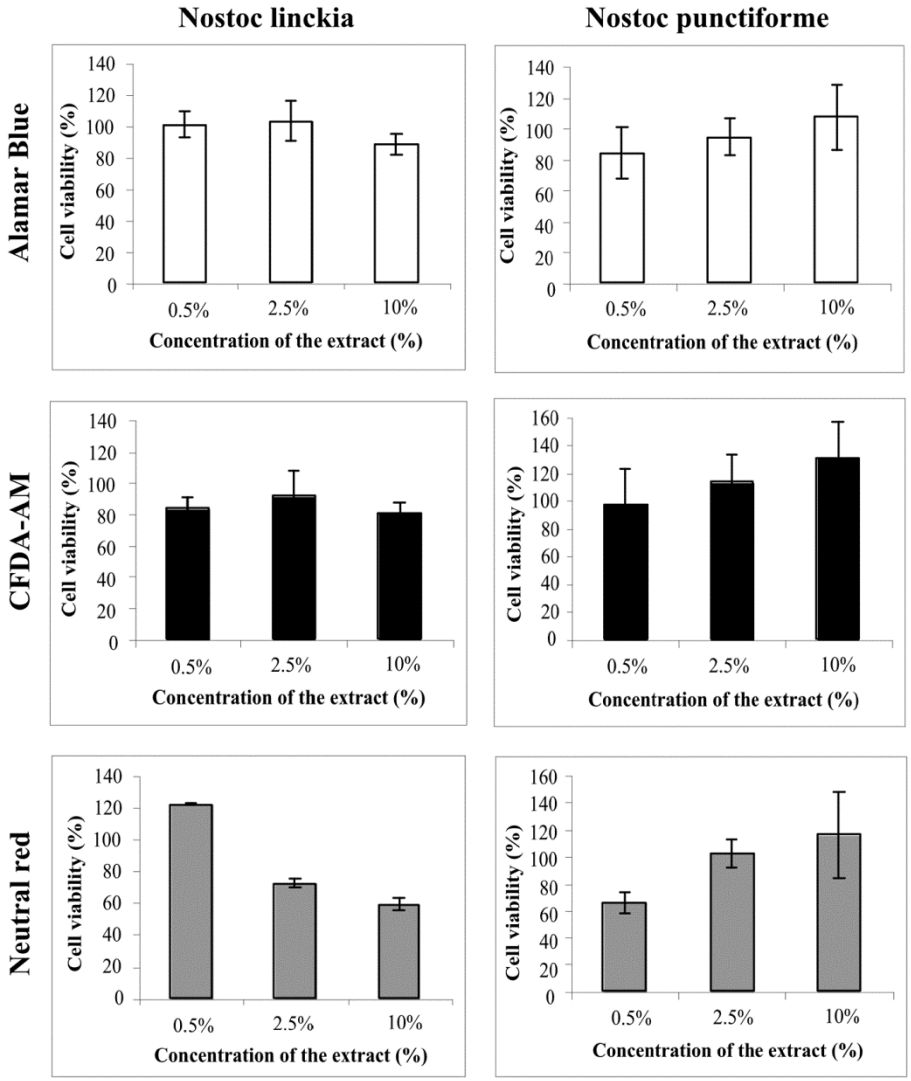

Figure 2. *Viability of RTgill fish cells treated with Nostoc extracts for 24h at 19°C. Viability was assessed using a mixture of the alamar Blue (upper) and CFDA-AM (middle) fluorescent indicator dyes as well as neutral red (down). Cells were exposed to equivalent concentration of suspended algal matter of 0.75 mg/ml (0.5% of extracts), 3.75 mg/ml (2.5% of extracts) and 15 mg/ml (10% of extracts). After exposure, alamar Blue, CFDA-AM and neutral red were applied as described in Materials and Methods. Unit readings were expressed as percentage (%) of the readings in the cultures receiving Millipore water as the control. Data are represented as mean values of triplicates ± SD.*

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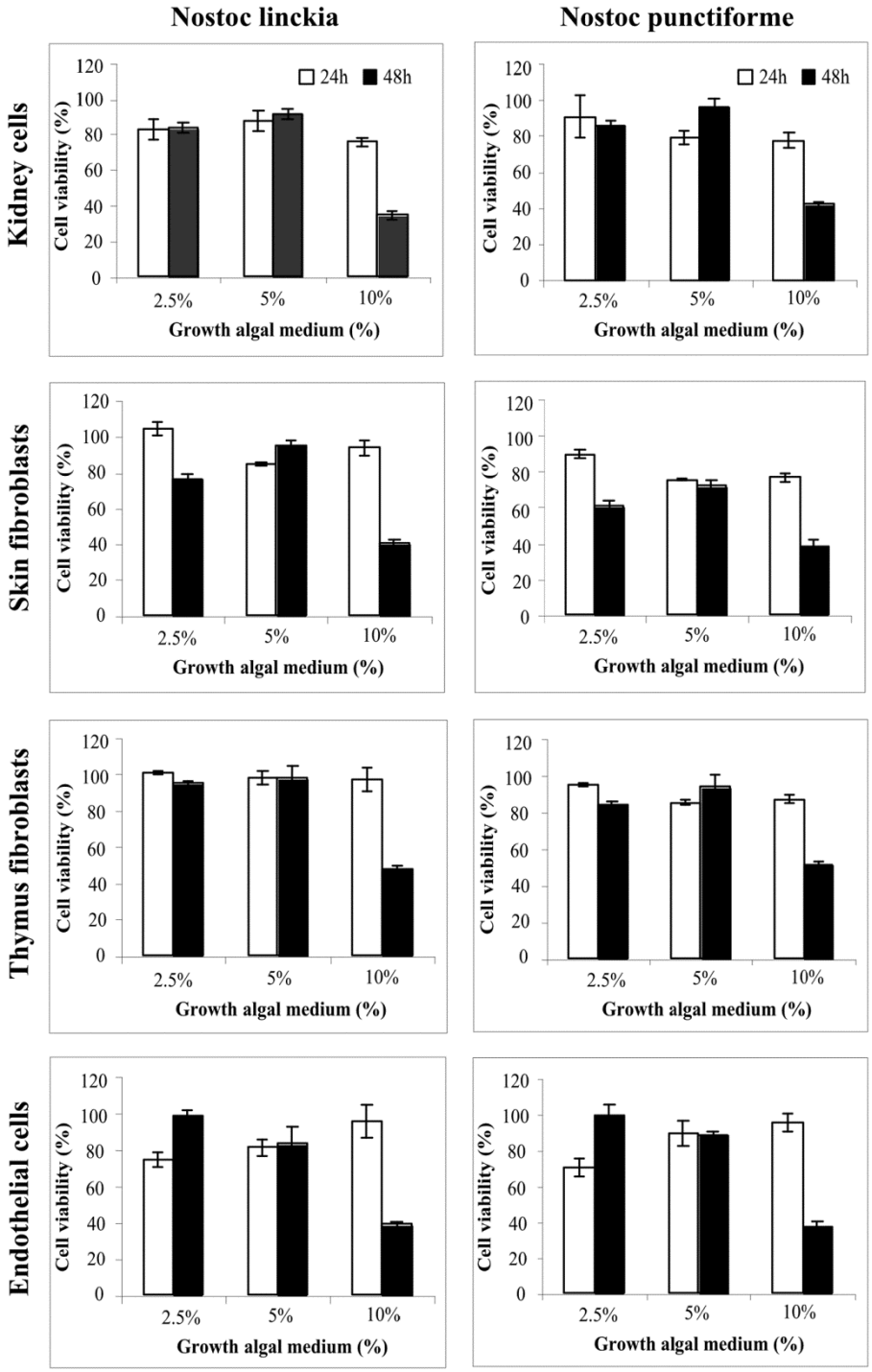

Figure 3. *Viability of mouse cell cultures treated with Nostoc growth media for 24h (white bars) or 48h (black bars) as determined by MTT assay. Cells were exposed by diluting the culture medium with 0.5%, 2.5% and 10% of the Nostoc growth medium. An equivalent percentage (%) of Z-medium (the medium in which the Nostoc species had been grown) was added to the control cultures. Data are represented as mean values of triplicates ± SD.*

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In vitro toxicity of Nostoc growth media

In order to determine whether investigated *Nostoc* species produce extracellular toxins, the media in which blue-green algae were grown for 14 days were tested as well. Both *Nostoc* growth media showed similar to the extracts effects. As illustrated in Figure 3, only highest concentration of growth media (10%) had significant cytotoxic effect on all mouse cultures after 48h of exposure. Treatment of mouse endothelial cells for 24h with *Nostoc* growth media showed effects similar to those observed in fish cells treated with *Nostoc* extracts.

Lower amounts of growth media induced a cytotoxic effect, while at higher concentration this effect disappears (Figure 3). Treatment of endothelial cells with *Nostoc linckia* or *Nostoc punctiforme* growth media for 48h resulted in dose-dependent cytotoxicity (Figure 3).

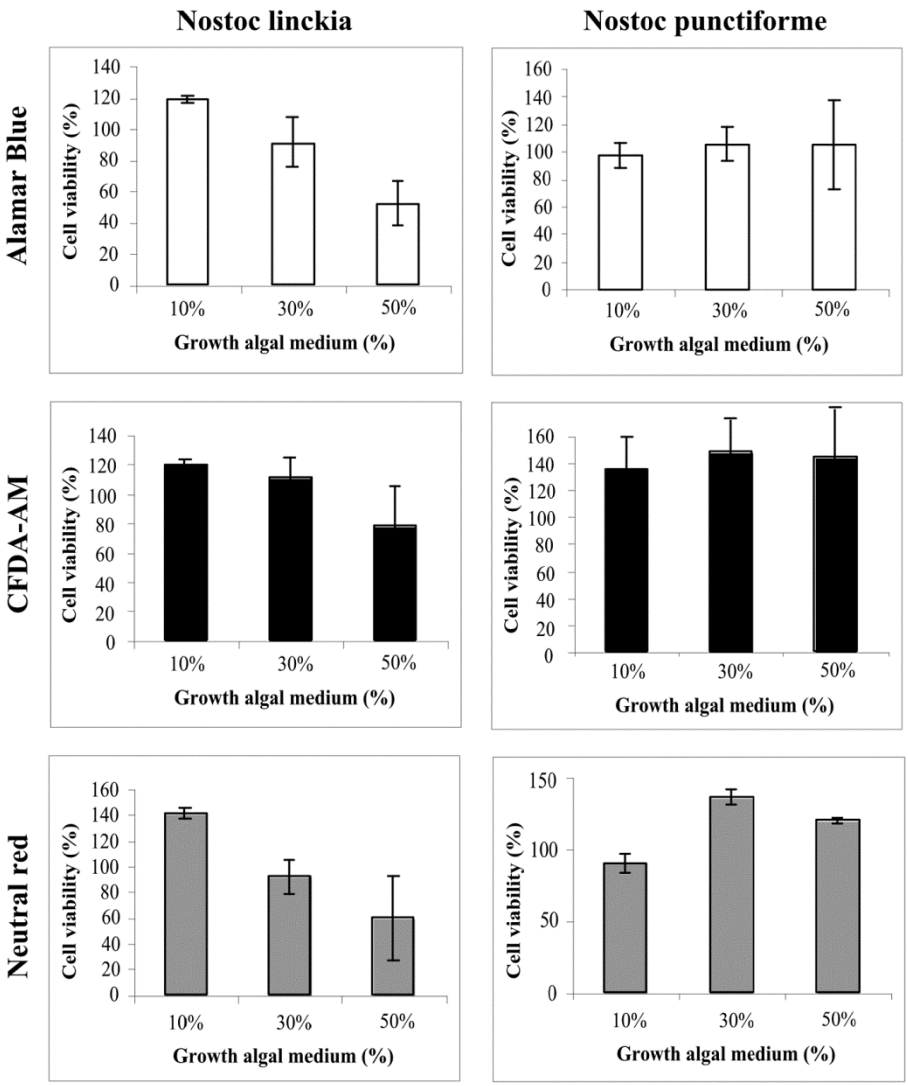

Figure 4. *Viability of RTgill fish cells treated with Nostoc growth media for 24h at 19°C. Viability was assessed using a mixture of the alamar Blue (upper) and CFDA-AM (middle) fluorescent indicator dyes as well as neutral red (down). Cells were exposed by diluting the culture medium with 10%, 30% and 50% of the Nostoc growth medium. An equivalent percentage (%) of Z-medium (the medium in which the Nostoc species had been grown) was added to the control cultures. After exposure, alamar Blue, CFDA-AM and neutral red were applied as described in Materials and Methods. Data are represented as mean values of triplicates ± SD.*

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RTgill cells were treated with 10%, 30% and 50% with growth medium for 24h. Data shown in Figure 4 indicate that after 24h of exposure to *Nostoc linckia* growth medium, cell viability dropped to 50-60% in a dose-dependent manner as measured by Alamar blue, CFSA-AM and neutral red assays.

The treatment of RTgill cells with *Nostoc punctiforme* growth medium caused effects similar to the extract of the same species. A weak decrease of cell viability at lowest concentration (neutral red test) and a stimulatory effect with increasing the concentration of growth medium were detected (Figure 4). At lowest concentration, this growth medium had a cytotoxic effect on mouse cells, being in the range 2-21% dependent from the type of cells (Figure 3).

Results from our *in vitro* tests showed that the mouse cells are more sensitive to toxic compounds than the fish cells. *Nostoc linckia* and *Nostoc punctiforme* produce different intracellular and extracellular compounds with different mode of action.

HPLC analysis

To further identify the toxic compounds, *Nostoc* extracts and growth media were analysed by HPLC using comparison of retention times to standards of cyanotoxins (Figure 5). HPLC was arranged to detect cyanotoxins from different groups (e.g. anatoxin-*a*, saxitoxins, MC-LR) by one run under ones and the same conditions. Figure 5A shows the HPLC chromatogram of a standard mixture including AnTx-*a*, STX and MC-LR. *Nostoc* extracts and growth media showed distinct HPLC profiles (Figure 5B,C, 5D,E). Both extracts as well as growth medium from *Nostoc linckia* showed peaks with retention time close to the neurotoxin AnTx-*a* (6.59-7.69 min). This peak was not found in *Nostoc punctiforme* growth medium (Figure 5E). There was a peak in both extract and growth medium prepared from *Nostoc linckia* with retention time 15.01–15.32 min (Figure 5B,D), which was not detected in *Nostoc punctiforme* samples (Figure 5C,E). Since a similar small peak was observed in the standard chromatogram (Figure 5A), we guessed that this is LPS contamination, which could be obtained very often in such samples. The additional HPLC run of LPS at the same conditions confirmed our hypothesis (Figure 5D, upper small chromatogram).

A considerable peak with retention time 34.21 min was detected in the *Nostoc punctiforme* extract (Figure 5C) but not in the *Nostoc linckia* extract (Figure5B), which can explain the stimulatory effect on fish cells (Figure 2). HPLC chromatograms showed the presence of microcystins (retention time = 47.83 min) in *Nostoc linckia* extract (Figure 5B) and *Nostoc punctiforme* growth medium (Figure 5E). The other detected peaks require more detailed identification steps.

The HPLC analysis confirmed the presence of cyanotoxins (even in low doses) in both extracts and growth media of the investigated *Nostoc* species.

ELISA analysis

To confirm the presence of cyanotoxins, we next tested the *Nostoc* extracts and media by commercially available ELISA kits for saxitoxins and microcystins. Neither group of toxins was detectable in the media. According to the saxitoxin ELISA, which has 10-30% cross reactivity to decarbamoyl saxitoxin, gonyautotoxins II, III, B1, C1 and C2, both *Nostoc* extracts contained minor levels of this group of toxins close to the detection limit of the ELISA (0.010 ppb). The microcystin/nodularin ELISA kit cross-reacts to microcystin LR, LA, RR, YR and nodularin. In our samples these toxins were detected in both extracts with concentration 0.05625 ppb.

Conclusion

The present study demonstrates that *Nostoc* species in addition to production of useful bioactive compounds with therapeutic action like cryptophycins, are able to produce other intracellular and extracellular toxic compounds as well as some typical cyanotoxins. Thus, the capability of separate cyanobacterial strains to produce potential hazard compounds has to be taken in account when such strains are used as a source of different bioactive agents with pharmaceutical potential.

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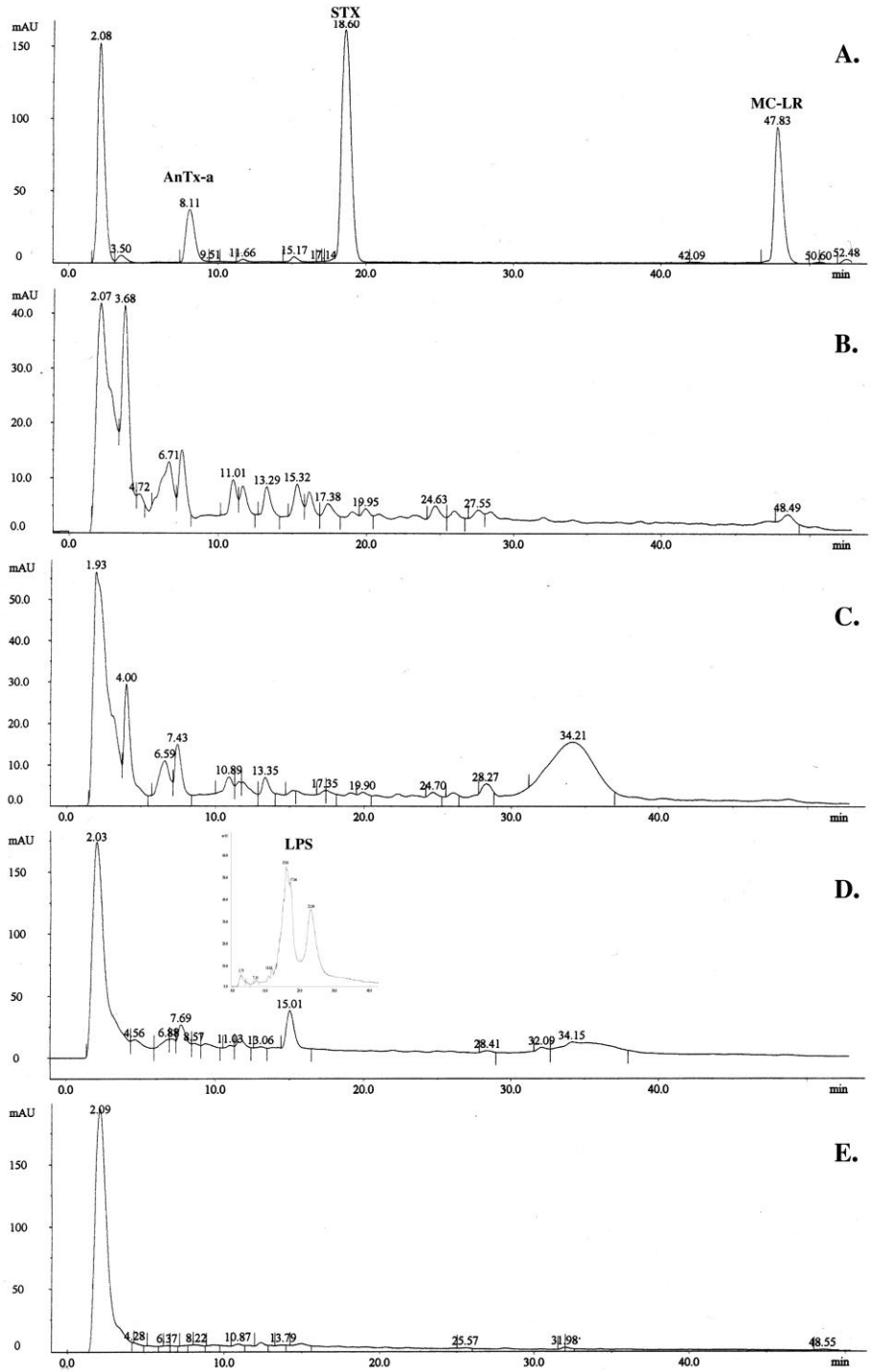

Figure 5. *HPLC chromatograms of (**A**) a mixture of standard cyanotoxins; (**B**) the extract obtained from Nostoc linckia; (**C**) the extract obtained from Nostoc punctiforme; (**D**) the growth medium of Nostoc linckia (upper small chromatofram represents LPS) and (**E**) the growth medium of Nostoc punctiforme. A volume of 200 µl of each sample was used for HPLC analysis under the conditions described in Materials & Methods.*

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